Regulation of trophoblast cell functions by intracellular signalling molecules and microRNAs

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Dedicated to my family

"Luna quieres ser madre

y no encuentras querer

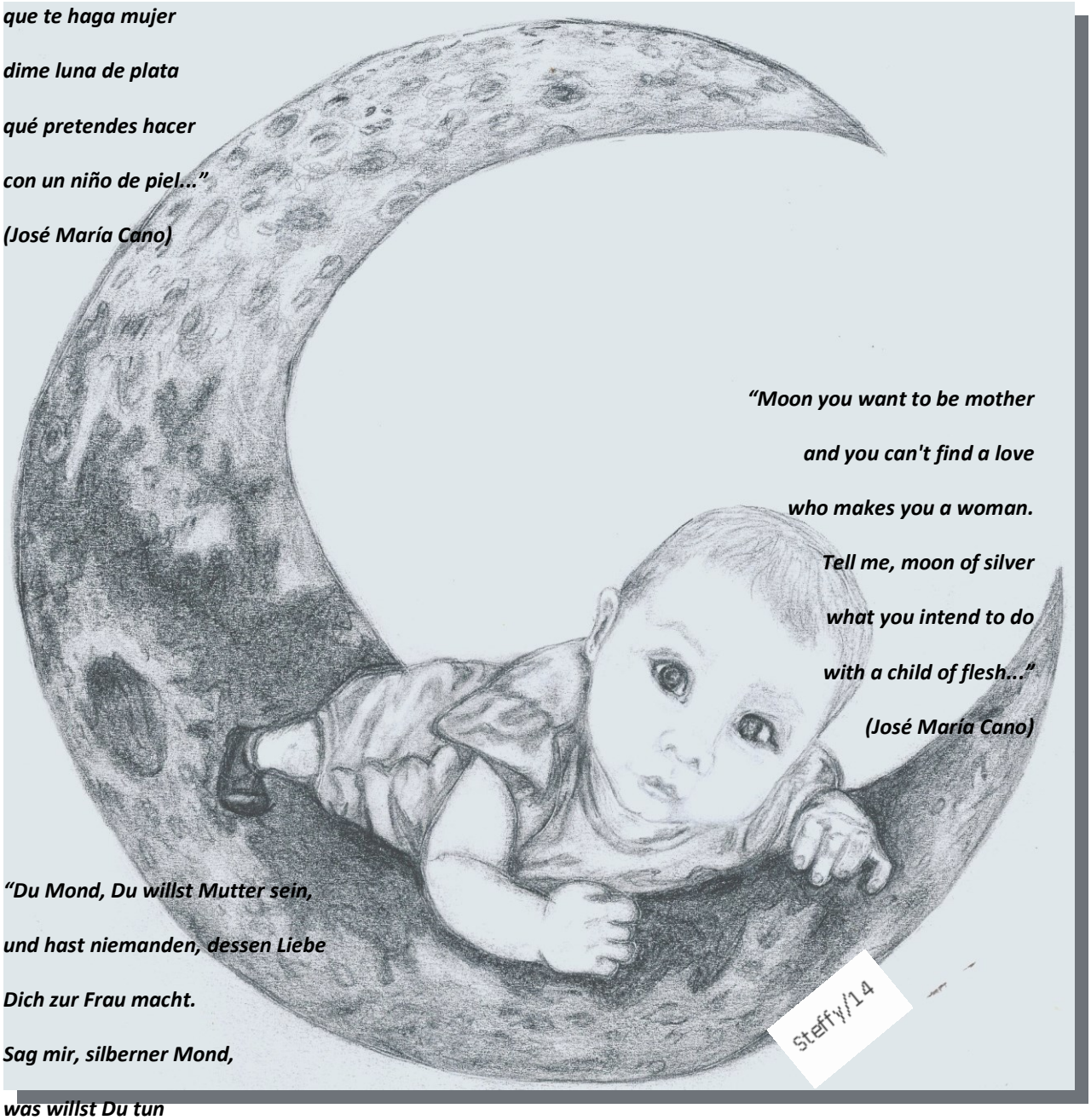
que te haga mujer

dime luna de plata

qué pretendes hacer

con un niño de piel..."

(José María Cano)



"Moon you want to be mother

and you can't find a love

who makes you a woman.

Tell me, moon of silver

what you intend to do

with a child of flesh..."

(José María Cano)

"Du Mond, Du willst Mutter sein,

und hast niemanden, dessen Liebe

Dich zur Frau macht.

Sag mir, silberner Mond,

was willst Du tun

mit einem Wesen aus Fleisch…"

(José María Cano)

Contents

List of abbreviations

AB	Acrylamid/ Bisacrylamid
ABC	Avidin/Biotinylated enzyme Complex
Ago 2	Argonaute 2
AP-2α	Transcription factor AP-2 alpha
APS	Ammoniumpersulfat
BrDU	5-bromo-2'-deoxyuridine assay
C14MC	Chromosome 14 miRNA cluster
C19MC	Chromosome 19 miRNA cluster
CO2	Carbon dioxide
CTR	Non-transfected cells
ddH2O	Doubly distilled water
EGF	Epidermal Growth Factor
EGFR	Epidermal Grown Factor Receptor
ETS2	ETS transcription factor
EMVs	Extracellular vesicles
EVT	Extravillous cytotrophoblast
EXOs	Exosomes
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDM	Gestational diabetes
GM-CSF	Granulocyte macrophage-colony stimulating factor
hCG	Chorionic gonadotropin
HGF	Hepatocyte Growth Factor
HLA-C	HLA class I heavy chain paralogues
HLA-E	HLA class I histocompatibility antigen, alpha chain E
HLA-G	Human leukocyte antigen G
hPL	Human placental lactogen
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IL-11	Interleukin-11
IL-2	Interleukin 2
IL-6	Interleukin-6
IUGR	Intrauterine growth restriction
JAK/STAT	Janus Kinase/Signal Transducer and Activators of Transcription
LBW	Low birth weight
LIF	Leukemia Inhibitory Factor
MAPK	Mitogen-activated protein kinase
MiRNA	MicroRNA
MPs	Microparticles
mRNA	Messenger RNA
MTS	Tetrazolium salt assay
MVs	Microvesicles

Nb2	rat lymphoma cells
NTA	Nanoparticle tracking analysis
OSM	Oncostatin M
PBS	Phosphate buffered saline
PE	Preeclampsia
PHA	Phytohaemaglutinin
PI3K/AKT	Phosphoinositol 3-kinase/Akt
PLC γ /PKC	Phospholipase C-γ/protein kinase C
Pre-SCR	Non-genomic negative controls
PVDF	Polyvinylidene fluoride
RISC	RNA-induced silencing complex
RT	Room temperature
RT-qPCR	Real-time reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfat
SGP	Stacking gel buffer
siRNA	Small interfering RNA
TBS-T	Tris-buffered saline containing Tween-20
TEMED	Tetramethylendiamin
TGP	Running gel buffer
TMB	Tetramethyl-benzidine
Tyk2	Tyrosine kinase 2
UTR	3’ untranslated region

Chapter 1/ Introduction

Pregnancy has commonly been viewed as a cooperative interaction between a mother and her fetus [1]. This interaction is carried out entirely by the placenta. It is defined as a complex organ that facilitates physiologic exchange between the developing fetus and its mother and it is fundamental for pregnancy success or failure [2].

The eutherian (mammalian) placenta varies dramatically among species due to high anatomical diversity, such as differences in placental shape, number of layers of trophoblasts, shape of maternal-fetal interdigitation, the contact between fetal and maternal tissues and their access to maternal blood flow [3, 4]. These complex structures have been topic of studies for nearly 20 years and many interpretations have been raised in order to understand tracing patterns of placental evolution [5, 6].

Actually, three main types of placental interface have been classified according to the extent of how the fetal tissue invades the wall of the uterus or the maternal vessels. There are epitheliochorial, endotheliochorial and haemochorial placentas (**Fig. 1**). Epitheliochorial placentation is considered to have three layers of maternal tissue separating the fetus from maternal blood and displaying poor invasiveness [3]. Important representatives are lemurs (lower primates), eastern moles (Scalopus), horses (Perissodactyla) and whales (Cetacea) [5]. The second type of placentation, endotheliochorial, is characterized by a partially invasive phenotype, in which only the endothelial wall of the maternal blood vessels separates the fetus from the maternal blood [3]. Carnivora is one of representatives of this group. Finally, haemochorial placentation features a range of properties that allow fetal tissues to be bathed directly in the maternal blood and includes a major invasion than the other two kinds of placentation which has the advantage of a more efficient nutrient uptake and waste elimination. However, this direct contact between maternal and fetal tissue implies an increased risk of maternal bleeding after delivery and a greater chance of fetal cell transfer to the maternal system [3, 4]. For instance, human and mouse placentas belong to the haemochorial type but the human placenta differs from mice in cell biology [7], endocrine system [8], immune system [9] and placental transport [10].

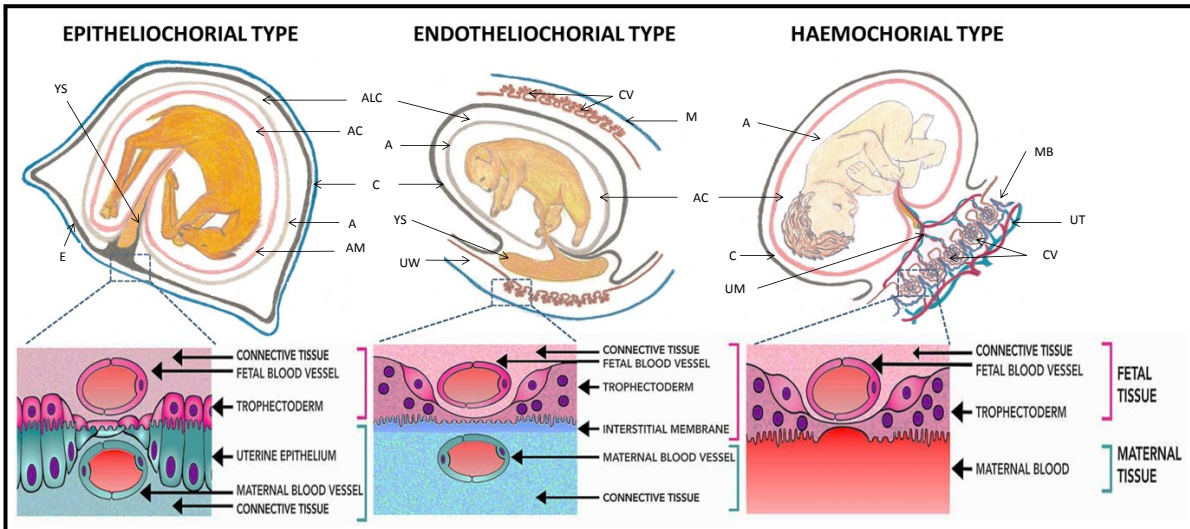


Figure 1. Models of placentation according to the extent of fetal tissue invasion into the wall of the uterus or the maternal vessels in eutherian species. YS: Yolk sac, E:Endometrium, ALC: Allantoic Cavity, AC: Amniotic cavity, C: Chorion, AM: Amnion, A: Allantois, UW: Uterine Wall, CV: Chorionic Villy, M: Myometrium, UM: Umbilical artery and vein, MB: Maternal blood supply in intervillus space, UT: Uterine artery and vein (Modified after [11]).

Due to differences to other species, the human pregnancy is considered to possess great plasticity and has been generating exciting questions for immunologists and reproductive biologists for nearly 60 years. For example, the immunological situation found during pregnancy is considered as a paradox caused by the fact that in pregnancy the semi-allogeneic fetus must be tolerated [12]. Another topic that produces fascination is the formative stages of placental development (from the time of implantation at 5 weeks of gestation). These are little understood because of the limited access to corresponding samples due to ethical and practical reasons leading to the metaphor of a “black box” for this period of placental development [13]. Finally, the comparatively inefficient human reproduction is a matter of great importance, in special for the assisted reproductive technologies. Fertility rates are only 20% per month and an additional third of all fertilized human embryos get lost during the early post-implantation period. For this reason, the human reproduction success is much lower than that of many other eutherian species, for which the estimates in primates, rodents and domestic animals are 70% [13-15]. Therefore, the time of implantation of the blastocyst and early development of the placenta is fundamental for understanding the success of human pregnancy.

1.1. Human implantation: "Implantation window"

After fertilization, the embryo initiates its fast division and enters the uterine cavity at the morula stage (around 4 days). Subsequently, it develops into a blastocyst and then hatches from the *zona pellucida*. The free-floating blastocyst is conducted at the uterine wall in order to transform the endometrium for pregnancy support. Once the blastocyst is in contact with the endometrial epithelium (apposition), the embryonic pole is oriented to the potential implantation sites and fetally derived cells (trophoblasts) penetrate between the luminal epithelial cells at the point of attachment (adhesion), invade through the basal lamina and penetrate deeply into the decidualized stroma until they engraft the maternal vasculature (invasion). The juxtaposition of both maternal-fetal layers remodels the endometrial spiral arterioles into low-resistance vessels that are unable to constrict and become enlarged and oedematous. Then, the luminal epithelium is restored over the implantation site so that embryo and placenta are completely contained covered by the uterine wall and become highly secretory. This well-defined period of menstruation cycle is called "implantation window" (**Fig. 2**) [13, 15, 16].

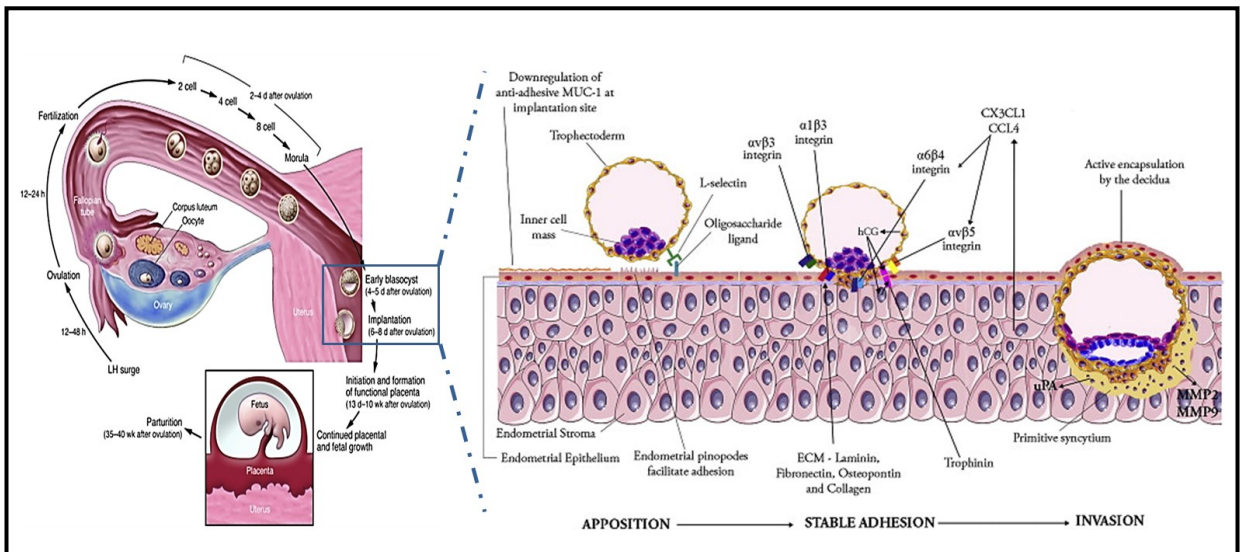


Figure 2. Schematic diagram of blastocyst implantation to endometrium. The implantation involves three key phases- apposition: attachment of the blastocyst to the endometrium; adhesion: stable attachment; and finally invasion between luminal epithelial cells. (Modified after [13, 17]).

Implantation induces three major consequences. First, the fetus gains direct access to its mother's arterial blood. Second, the volume of blood reaching the placenta becomes largely independent of control by the local maternal vasculature. Third, the placenta is able to release hormones and other substances directly into the maternal circulation for fetal benefit, such as chorionic gonadotropin (hCG) and human placental lactogen (hPL) [1].

An inappropriate invasion results in pregnancy disorders, like preeclampsia (PE) and increases the risk of spontaneous abortion. Therefore, a successful implantation requires three key factors. First of all, an embryo competent to implant; second, a receptive endometrium as a result of the sequential exposure of the endometrium to oestrogen followed by progesterone, and finally, a coordinated dialogue between endocrine, paracrine and juxtacrine modulators that depends on cellular and molecular crosstalk between the blastocyst and the receptive endometrium. This dialogue is mediated by local factors, including hormones, cytokines, prostaglandins and adhesion molecules, and results in local changes in the epithelium which permit the attachment of the embryo [15].

1.2. Trophoblast Cells and Human Choriocarcinoma

Trophoblast cells are a very heterogeneous cell type originating from a stem cell population which continuously proliferate and differentiate into extravillous trophoblasts or syncytiotrophoblast. They display numerous functions. A major function is anchoring the conceptus in the uterus, which trophoblast cells fulfil due to their ability to invade the maternal decidual tissue. A similar capacity can be also observed in cancerous tumor cells providing certain analogy between these two cell types. In other words, trophoblast cells use similar molecular mechanisms as tumors for migration and invasion [18, 19]. However, trophoblast invasion is limited in time and space while in cancerous tumor cells it is de-regulated [20].

Another joint characteristic of trophoblast and tumor cells is the capacity of protecting themselves from the maternal or host immune system and thus to escape from potentially detrimental immunological responses from the mother or the host [16]. These properties are fundamental to consider trophoblast cells as an optimal candidate for studying invasive behavior and the molecular mechanisms applicable to pregnancy and cancer. Based on this

knowledge, it may be possible in future to define molecules potentially useful in cancer prevention or treatment as well as in pregnancy disorders. The underlying intracellular mechanisms and most signaling pathways remain only partially understood.

1.3. Cytokines and growth factors

Cytokines are a family of specific extra-cellular ligands, which use several distinct intracellular signaling pathways. They can control trophoblast behavior by either modulating proliferation or migration or inducing trophoblast cells to differentiate into a non-invasive phenotype [16, 21]. Cytokines stimulate biological responses by binding to, and activating a family of structurally and functionally conserved cytokine receptors [22]. This event triggers receptor homodimerization [23], heterodimerization or oligomerization of receptor subunits [24], or it induces a conformational change in preformed receptor dimers [25], which, e.g., results in the activation of Janus kinases (JAKs).

Growth factors correspond to a family of secreted signaling proteins that act as initiators of signaling cascades in cells by paracrine and endocrine interactions. They are capable of inducing a variety of cellular processes and can be classified according to both structural and evolutionary associations, ordering them into larger families of proteins [26].

Several cytokines and growth factors have been studied in the field of reproductive medicine some of which play a pivotal role. They include Interleukin-6 (IL-6), Oncostatin M (OSM), IL-11, Hepatocyte Growth Factor (HGF), Leukemia Inhibitory Factor (LIF), Granulocyte macrophage-colony stimulating factor (GM-CSF) and Epidermal Growth Factor (EGF). These cytokines use the Janus Kinase/Signal Transducer and Activators of Transcription (JAK/STAT) pathway, and thereby, influence cell invasion, proliferation, differentiation, migration and apoptosis [16, 27].

1.3.1. Epidermal Growth Factor (EGF)

Epidermal Growth Factor (EGF) plays an important role for embryo implantation, trophoblast differentiation and endocrine functions of the placenta [28, 29]. Several studies based on

different cell models have demonstrated that EGF influences positively or negatively a variety of fundamental cell properties like proliferation [30-32], differentiation [32], inhibition of apoptosis [33-36], motility [37-39], secretion [40, 41], angiogenesis [29] and invasion/ migration [37, 42-44]. However, the role of EGF on regulation of trophoblast behavior and the intracellular mechanisms responsible for these effects remain poorly understood.

EGF binds to its cell surface receptor (EGFR) where it induces receptor dimerization and cross-phosphorylation. This leads to assembling of a signaling complex composed of cytoplasmic enzymes and adaptor proteins. The subsequent dissociation of this complex releases activated effector and adapter proteins into the cytoplasm where they stimulate a variety of intracellular pathways [45]. These cascades include phosphoinositol 3-kinase/Akt (PI3K/AKT), mitogen-activated protein kinase (MAPK), Phospholipase C-γ/protein kinase C (PLCγ /PKC), transcription factor AP-2α, ETS2 and the JAK/STAT cascade [46, 47] (**Fig. 3**). Cross-talks between the JAK/STAT and MAPK pathways in trophoblastic cells have been described [27, 48].

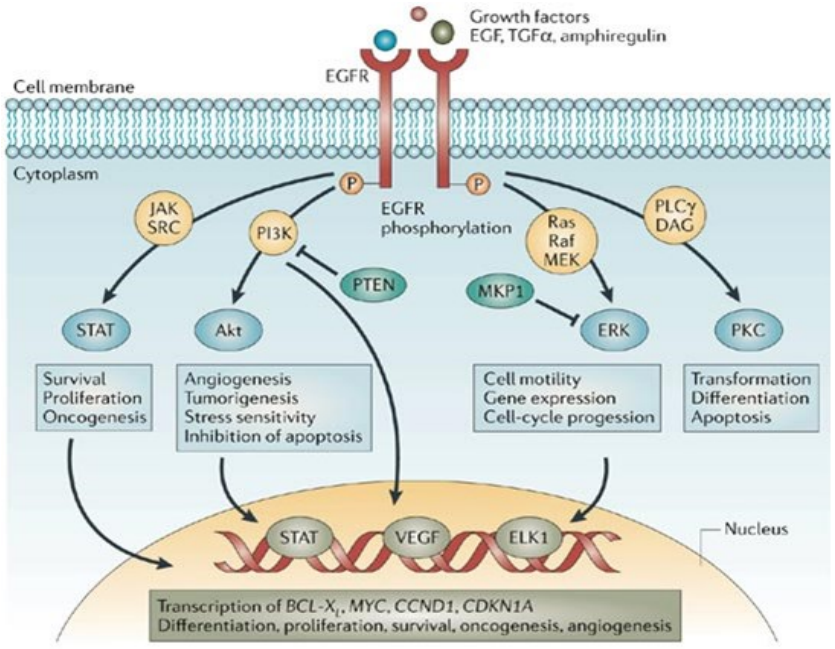


Figure 3. The main downstream signaling pathways regulated by EGFR. Upon binding of its ligands, EGFR homodimerizes leading to phosphorylation of many tyrosine residues localized in the carboxy-terminal tail of EGFR. Phospho-EGFR is then able to recruit adaptor proteins. Important signaling pathways regulated by EGFR are represented with important functions highlighted in coloured boxes. The signaling cascade does not include all the known components of a given pathway, and cross-talk is not shown for clarity (Taken from [49]).

1.4. Classical signaling pathways: JAK/STAT pathway

The JAK/STAT signal transduction pathway is composed by five steps. Initially, the cytokine and receptor binding leads to the juxtaposition of receptor associated JAKs. Then, these JAKs become phosphorylated and activate each other and the inner receptor ligand (2nd step). Subsequently, STATs bind to inner receptors to be phosphorylated and activated by JAKs (3rd step). When STATs are activated, they dissociate from the receptor and form homo- and heterodimers. Finally, these dimers translocate into the nucleus and promote transcriptional activation of cytokine inducible genes (4th and 5th) (**Fig. 4**). Thus the JAK/STAT cascade provides a direct mechanism to translate an extracellular signal into a transcriptional response [16, 50, 51].

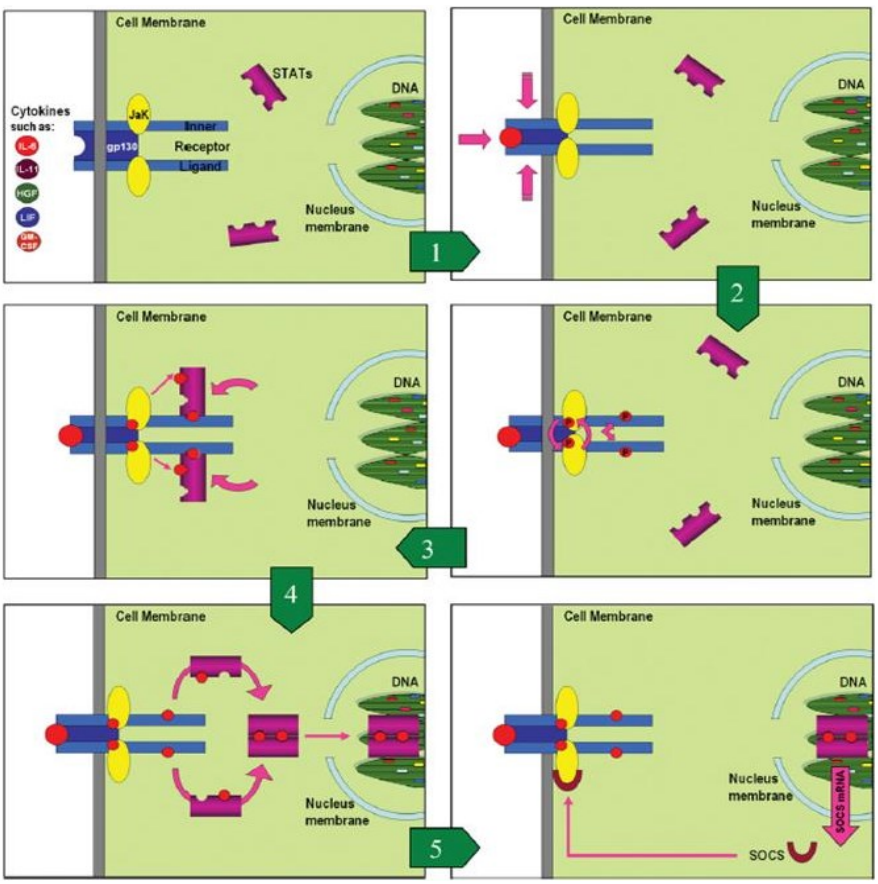


Figure 4. JAK/STAT pathway. 1). Cytokine binding leads to receptor aggregation; 2) JAKs phosphorylate and activate each other and inner receptor ligand; 3) STATs bind to inner receptor ligand to be activated by JAKs; 4) activated STATs disassociate to from homo- and heterodimers and translocate into the nucleus to accelerate transcription of target proteins; 5) one target protein family are SOCSs which limit further STAT activation by binding to JAKs (Taken from [16]).

The family of JAKs is characterized by large tyrosine kinases that include JAK1, JAK2, JAK3 and Tyk2. These proteins are cytoplasmic tyrosine kinases that consist of seven different domains and whose functions are complex and still being elucidated [51-53]. STATs are latent cytoplasmic transcription factors that can interact with some nuclear factors and coactivators, which increase the range of transcriptional responses in which STATs and its isoforms can participate. The STATs family comprises seven members in mammals: STAT1 to 6, including STAT5A and STAT5B. They can be activated by partially overlapping sets of cytokines but different STAT molecules have non-redundant biological roles [51, 54, 55]. STAT3 has been extensively studied in pregnancy because of its relevance in the control of trophoblast proliferation and invasion [48, 56]. More recently, STAT5A and B have been investigated because their dysregulation is associated with pregnancy pathologies. STAT5A and STAT5B share 96% of their sequence but are encoded by two different genes on chromosome 17 [57, 58]. Each protein has its own function but they can also work synergistically [59, 60]. STAT5A is important for mammary gland development, augmenting milk protein expression on prolactin induction [58, 59], and its deficiency results in a severe loss of mammary gland development in mice [61]. On the other hand, STAT5B controls growth hormone signaling and regulates expression of sexually dimorphic genes which control male/female size differences in mice [59]. Additionally, lack of STAT5B is associated with early abortions that are due to reduced progesterone levels during midge-station. This effect is exacerbated in the phenotype of mice lacking both STAT5 proteins [61].

JAK/STAT pathway was discovered roughly 20 years ago and since then, many studies have revealed the important role of STATs in several biological responses, including cell proliferation and invasion. However, the knowledge about that is not complete [51, 55]. Moreover, the well characterized JAK/STAT system may provide a better understanding of the physiological mechanisms of invasion and its control and may serve as a valuable paradigm for studying the architecture of gene regulatory networks. The discovery of untranslated or non-coding RNAs, such as microRNAs, provide an opportunity to elucidate their roles in such networks. These microRNAs can act as downstream effectors of the JAK-STAT pathway and/or affect signaling by regulating the expression of JAK-STAT components. The dialogue between JAK/STAT cascade and microRNAs has already emerged in basic cell biology and human diseases, and may lead to novel molecular biomarkers and

pharmacological strategies to interfere with cellular processes and prevention of diseases. [62].

1.5. MicroRNAs

The study of epigenetics has attracted attention in different fields, such as human reproduction and has provided important novel interpretations of genetic research. Epigenetics has different meanings; one of them was defined by Conrad Waddington in 1939 as regulation of gene expression in a heritable manner that does not involve any modifications [63]. Since this time more meanings have been generated for different fields of science, however most of the definitions are connected to molecular biology and are similar to the definition of epigenetics as "the study of changes in gene expression that are not caused by changes in the DNA sequence". Four main mechanisms are considered as epigenetic processes: DNA methylation, genomic imprinting, histone modification, and non-coding RNAs, specifically through microRNAs (miRNAs) [64].

MiRNAs are small, single-stranded RNA molecules of approximately 20-24 nucleotides in length, which act as translational repressors by either degrading or inhibiting translation of messenger RNA (mRNA) targets. Given the large number of miRNAs annotated in the human genome, 30%–80% of human genes are predicted to be influenced by miRNAs [65]. However, it is important to mention that miRNAs are not complementary to only one specific mRNA but regulate simultaneously more than one gene. Furthermore, different miRNAs can target the same mRNA and have similar biological functions [66]. They can control numerous cellular processes including metabolism, cell proliferation, apoptosis and differentiation in almost all cells and they are involved in processes associated with establishment and maintenance of pregnancy including preparation of the endometrium for implantation, control of genes associated with inflammatory responses and regulation of immune tolerance-associated genes, such as human leukocyte antigen G (HLA-G) [67, 68].

MiRNAs seem to have an important regulatory function in eutherian organisms and some of them have been classified as human-specific microRNAs. The search for orthologs of 1733 annotated human mature miRNAs across 11 species by BLAST revealed 10 miRNAs which are human-specific and 12 further miRNAs with human-specific seed sequences [67, 69].

1.5.1. MicroRNA biogenesis

MiRNAs are transcribed from primary transcripts, which are then processed to mature miRNAs in two consecutive maturation steps. First, the pri-miRNAs are cleaved by the nuclear enzyme Drosha to form hairpin pre-miRNAs (70nt) and then, they are exported to the cytoplasm through Exportin-5. In the cytoplasm, the pri-miRNAs are cleaved by the enzyme Dicer to form mature miRNAs (19-24 nt). Finally, the mature miRNA is incorporated into a RNA-induced silencing complex (RISC), where it directly interacts with a member of the Argonaute 2 (Ago 2) protein family. The miRNA guides such protein complexes to partially complementary target sites, which are typically located in the 3' untranslated region (UTR) of mRNAs leading to inhibition of gene expression. If alignment is partial complementary, the alignment leads to transcriptional repression of the target mRNA, while complete complementary, the cascade ends in cleavage of the target mRNA [66, 70] (**Fig. 5**).

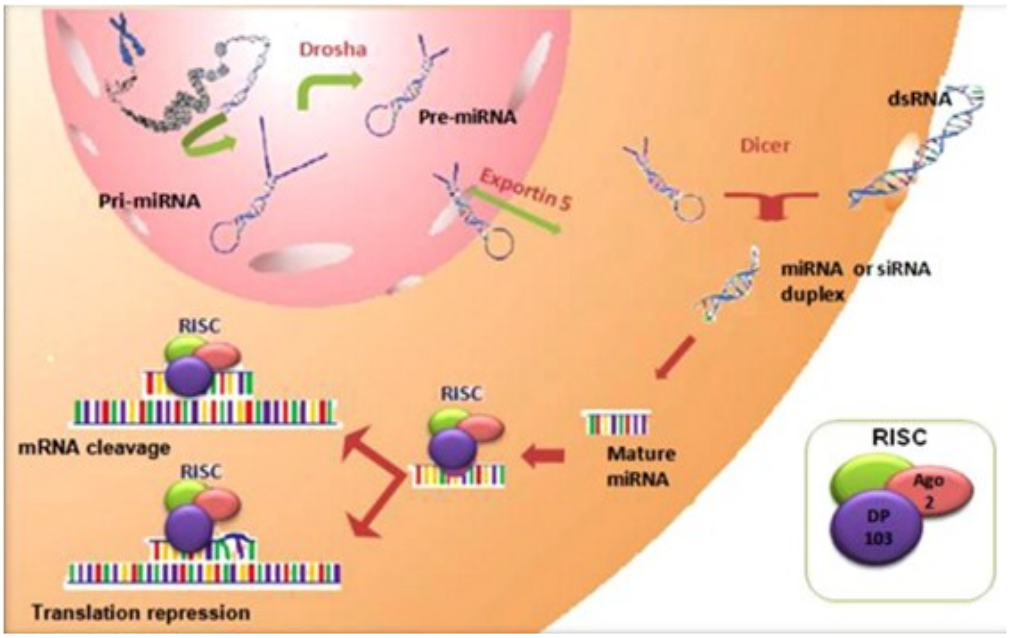


Figure 5. MicroRNA biogenesis. Inside the nucleus, pri-miRNAs are cleaved by Drosha to pre-miRNAs and transported into the cytoplasm by Exportin 5 (green arrows). The subsequent cascade is shared with exogenous siRNA (red arrows). Processing by Dicer results in mature miRNA or functional siRNA which bind to RISC and to complementary RNA sequences. Two major enzymes involved in the RISC complex are Ago2 and DP130. Complete complementary induces degradation and partial annealing leads to translational repression (Taken from [66]).

MiRNA activity and abundance is regulated on various levels ranging from transcription and processing to target site binding and miRNA stability [70]. For instance, miRNA biogenesis is regulated at several steps. First by Dicer processing and Ago2 protein complex formation, then in unwinding of the miRNAs within the RISC, loop size-dependent recognition and processing by Dicer, sequence complementarity between the miRNA and the target mRNA at the seed region (nucleotides 2-8 of the miRNA), duplex stability in regions outside the seed region, and finally, the presence of DNA Alu elements in the vicinity [71]. Additionally, each step is also regulated by common or cell-specific signaling, such as epidermal growth factor receptor (EGFR) signaling. EGFR phosphorylation of Ago2, which is enhanced by hypoxia, attenuates its interaction with Dicer, thereby reducing miRNA processing to mature miRNAs [72].

1.5.2. Placental miRNA

Placenta miRNA profiles have been studied in tissues, cell lines and maternal plasma. Most placenta specific miRNAs are localized within three miRNA clusters: chromosome 14 miRNA cluster (C14MC), chromosome 19 miRNA cluster (C19MC) and the miR-371-3 cluster (**Fig. 6**).

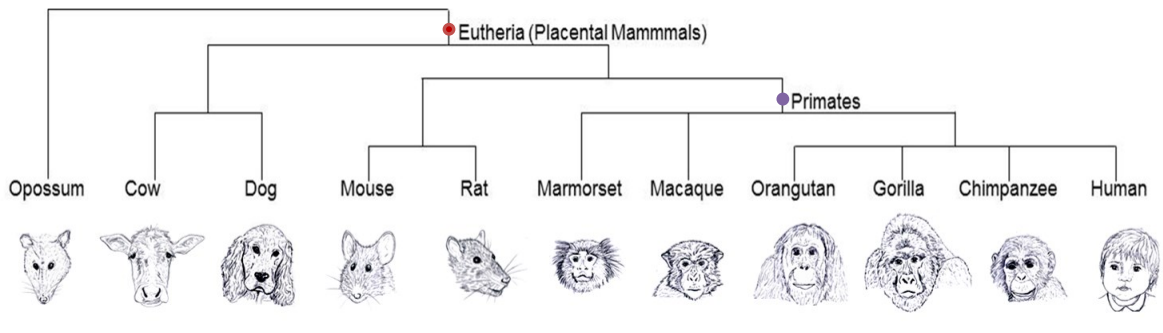


Figure 6. Orthologs of precursor miRNAs of pregnancy related clusters: (C14MC) is uniquely present in eutherian species (red circle), chromosome 19 miRNA cluster (C19MC) and the miR-371-3 cluster are just expressed in primates and conserved in humans (purple circle) (Modified after [67]).

However, some miRNAs are not present in those clusters but relevant during pregnancy. One of them is miR-141, a representative of the miR-200 family (**Fig. 7B**) that has been associated with nasopharyngeal and ovarian carcinomas [73]. This miRNA has been detected in maternal plasma and is considered as a potential pregnancy biomarker [74]. MiR-141 might be useful for monitoring regular pregnancy progress and evaluating the status of the placenta [75]. However, the exact role of miR-141 has not been analyzed so far.

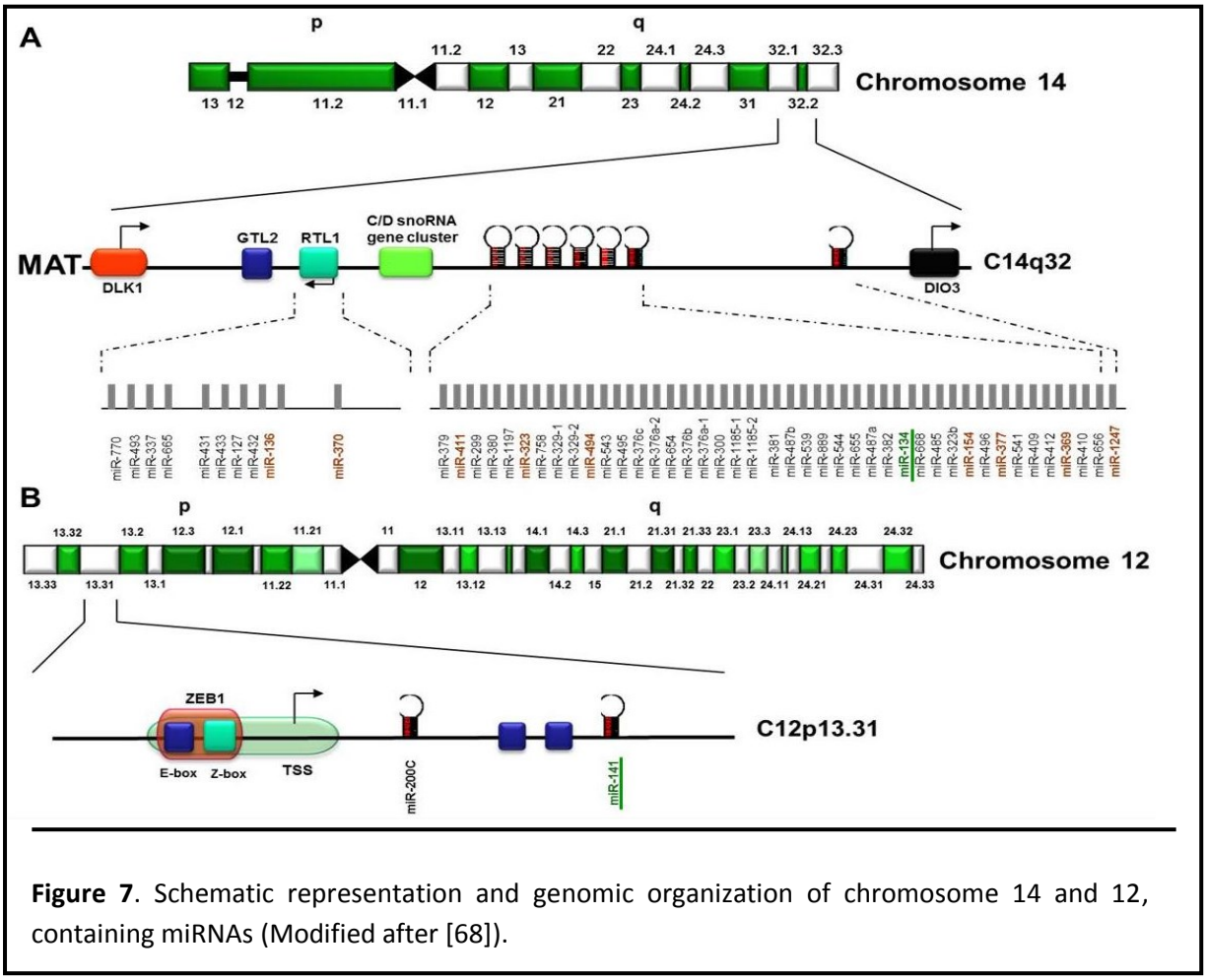


Figure 7. Schematic representation and genomic organization of chromosome 14 and 12, containing miRNAs (Modified after [68]).

Expression of miRNAs from the mentioned clusters changes during pregnancy and their expression differ among placentas from patients with disorders, such as PE, compared to normal pregnancies. Thus, they represent potential biomarkers for the respective pathologies. Remarkably, importance of these clusters in pregnancy might be due to their localization within imprinted genes involved in human embryonic development and playing important roles in the regulation of cellular differentiation and fate [68].

1.5.2.1. Chromosome 14 miRNA cluster (C14MC)

The large chromosome 14 miRNA cluster (C14MC), also referred to as the Mirg cluster [76] or the miR-379/miR-410 cluster [77] is located at the imprinted DLK-DIO3 domain on the human 14q32 chromosomal interval [77-79]. C14MC comprises 54 miRNAs only expressed from the maternally inherited chromosome, mostly in brain and placental or embryonic tissues [80, 81] and is uniquely present in eutherian species (the placental mammals) [76, 80] (**Fig. 7A**). Recent studies have revealed that C14MC precursor sequence appeared approximately 100 million years ago from an early mammalian ancestor prior to the radiation of Metatheria (marsupial) or Prototheria (monotreme) [81] by tandem duplication of the ancient sequence [79, 81]. Moreover, this cluster has remained similar without significant structural changes in all eutherian species suggesting that it might coordinate important biological roles in this lineage, like neurogenesis, embryonic development, transcriptional regulation and RNA metabolism which support the idea that the evolution of placental mammals was facilitated with this cluster [80].

Numerous miRNAs have been investigated and the results suggest that their dysregulation is connected to different diseases including tumors. Furthermore, the fact that C14MC is expressed abundantly in the normal placenta suggests that its alteration may be related to obstetric disorders such as preeclampsia. Many studies have shown evidence for preeclampsia-related down-regulation of miRNAs. These results may provide a basis for the development of clinical tools for diagnosis, treatment and prognosis of preeclampsia [67].

MiR-134, a member of C14MC, has been studied in mouse embryonic stem cells and this miRNA was identified as a potential regulator of dendritic spine volume and synapse formation in mature hippocampal neurons in vitro [82]. Mir- 134 also promotes proliferation of lung cancer cells [83] and is highly expressed in head and neck squamous cell carcinoma [84]. In a study on miRNA expression profiles of trophoblastic cells, we found that the expression of miR-134 is up-regulated in HTR-8/SVneo cells compared with JEG-3 cells, supporting the potential importance of miR-134 in pregnancy [75]. Recently, studies on cell-free serum and plasma samples from human volunteers using small RNA sequencing of cDNA libraries showed that miR-134 is specifically associated with pregnancy [85]. However, the specific functions of miR-134 in human pregnancy still remain unclear.

1.5.2.2. Chromosome 19 miRNA cluster (C19MC) and miR-371-3 cluster

Both of these clusters are encoded on chromosome 19. C19MC consists of 46 miRNA genes and is exclusively expressed in primates and conserved in humans, while orthologs of miR-371-3 cluster also can be found in other eutherian mammals including mouse. C19MC originated from a common ancestor, which might be a member of the miR-371-3 cluster [68].

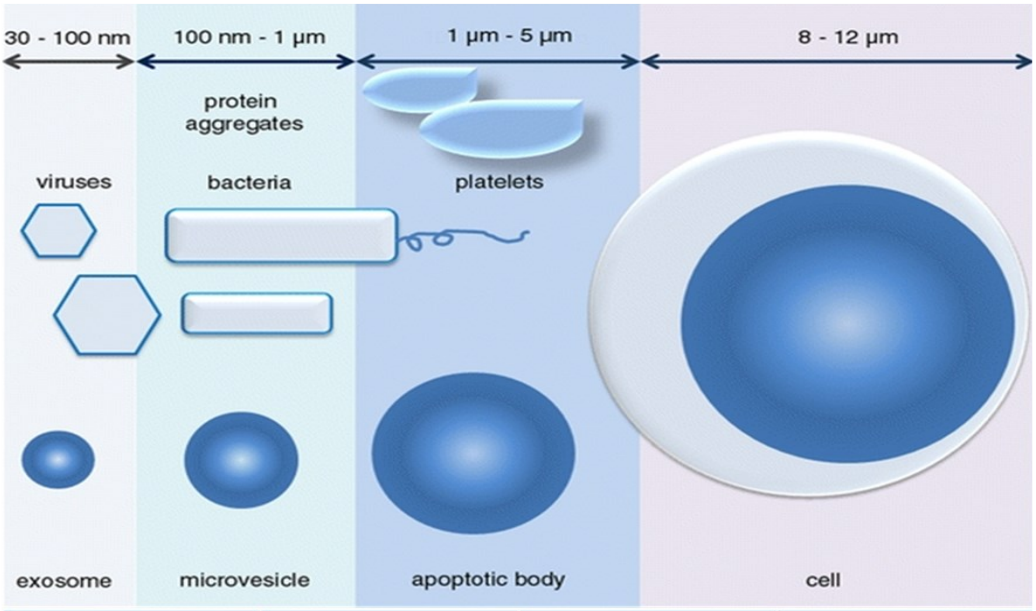
The C19MC and miR-371-3 cluster are predominantly expressed in the reproductive system, placental tissues and in stem cells [67]. Several groups have shown that these microRNAs are comparatively less intensively expressed in first trimester of pregnancy, but highly expressed in third trimester trophoblasts and in choriocarcinoma cell lines (JEG-3, ACH-3P and AC1-M59). After delivery, they are eliminated from the maternal blood. In contrast to the expression of C19MC, C14MC members decrease during the course of pregnancy [67, 71].

1.6. Cell-derived vesicles: Microparticles and Exosomes

In recent years, circulating extracellular miRNAs have attracted attention. Some miRNAs are stably detectable outside of cells in plasma and other body fluids of humans and these molecules appear to mediate cell–cell communication [86-89]. Additionally, secreted miRNAs are relatively well protected from digestion by RNase, conferring stability on the circulating miRNAs [90, 91]. Several studies have shown that extracellular miRNAs can be actively secreted into cell-derived vesicles such as microvesicles or exosomes for communication with adjacent or far distant cells [89]. The secreted miRNAs packaged in extracellular vesicles (EMVs) can be delivered into recipient cells and act as physiologically functional molecules to exert gene silencing through a similar mechanism as endogenous miRNAs [92]. Based on these observations it may be suggested that the intercellular transfer of extracellular vesicles represents a third mechanism for intercellular communication, different to direct cell-cell contact or transfer of secreted molecules [93].

The origin and nomenclature of extracellular vesicles has spread through the literature and different forms for called have been used [93]. EMVs are often divided into apoptotic debris, microvesicles (MVs)/microparticles (MPs) and exosomes (EXOs) (**Fig. 8**). Microvesicles are

phospholipid vesicles with a diameter of 100 to 1000 nm that are released by budding of the plasma membrane (ectocytosis) upon cell activation or apoptosis. Exosomes are preformed vesicles of endocytic origin with a diameter of less than 100 nm that are stored intracellularly in multivesicular bodies and are actively released by exocytosis from their paternal cells when these endosomal compartments fuse with the cell plasma membrane.



Feature	Exosomes	Microparticles	Apoptotic bodies
Size	50-100nm	100-1000 nm	Up to 4000 nm
Sedimentation	100 000 g	20 000 g	16 000 g
Origin	Multivesicular, internal compartments	Plasma membrane	Cellular fragments
Release	Constitutive and/or cellular activation	Cellular activation and early apoptosis	Terminal apoptosis
Annexin V binding capacity	No/low Annexin V binding capacity	High Annexin V binding capacity	High Annexin V binding capacity
Marker proteins	Tetraspan protein CD63	Integrins, selectins, otrher antigens of parental cell	Histones

Figure 8. Characterization of extracellular vesicles (EMVs). While exosomes share size distribution with viruses, microparticles overlap in size with bacteria and protein aggregates (e.g., immune complexes). Both, apoptotic bodies and platelets fall into the size range of 1–5 µm (Modified after [94, 95]).

Additionally to their size and origin, EMVs differ with regard to their membrane proteins and mode of release [94]. Despite the continuous effort to characterize and isolate EMVs, differences in properties such as size, morphology, buoyant density and protein composition seem to be insufficient for a clear distinction [93, 96]. Typical methods to separate EMVs are

ultracentrifugation, density-gradient centrifugation, gel chromatography or fluid-based field flow fractionation, with purity verified by nanoparticle tracking analysis (NTA) [97]. However, those methods have substantial limitations for discriminate between EXOs and MVs, suggesting that only when we are able to interfere with the molecular machineries required for EMV formation and cargo will be perfectly determined [93].

When the EMVs reach recipient cells, their molecular cargo may alter their phenotype and behavior. However, due to the different biogenesis between EXOs and MVs, they may use different ways to load regulatory RNAs. Some authors assume that exosomes are beneficial to normal pregnancy whereas microparticles may be harmful [98].

Functions of EMVs in physiological and pathological processes depend on the ability of EMVs to interact with recipient cells to deliver their contents. Target cell specificity for binding of EMVs is likely to be determined by adhesion molecules, such as integrins, that are present in EMVs. After binding to recipient cells, EMVs may remain stably associated with the plasma membrane or dissociate, directly fuse with the plasma membrane, or be internalized through distinct endocytic pathways. Endocytosed vesicles may then fuse with the delimiting membrane of an endocytic compartment or be targeted to lysosomes for degradation (**Fig. 9**). Both forms, fusion or endocytosis, result in the delivery of protein and RNA into the membrane or cytosol of the target cell, potentially resulting in cell activation, phenotypic modification and reprogramming of cell function [93, 94].

Several studies have reported the presence of EMVs from human placenta [98]. Exosomes appear to be released mainly during the first trimester of pregnancy whereas MVs can be recovered from the second trimester and their concentration increases as pregnancy progresses [99]. This difference in the time-dependent occurrence might reflect different biological functions in the placenta. Exosomes contain the immunosuppressive molecules HLA-G involved in the immune tolerance toward the fetus whereas MVs can contribute to the pro-inflammatory state required for normal pregnancy. Changes in the balance between these EMVs may therefore be critical for the development of the maternal syndrome [98, 99]. Therefore, circulating extracellular miRNAs may be potential noninvasive biomarkers for diagnosis, prevention or treatment of cancer and different pregnancy-related diseases as preeclampsia [100, 101].

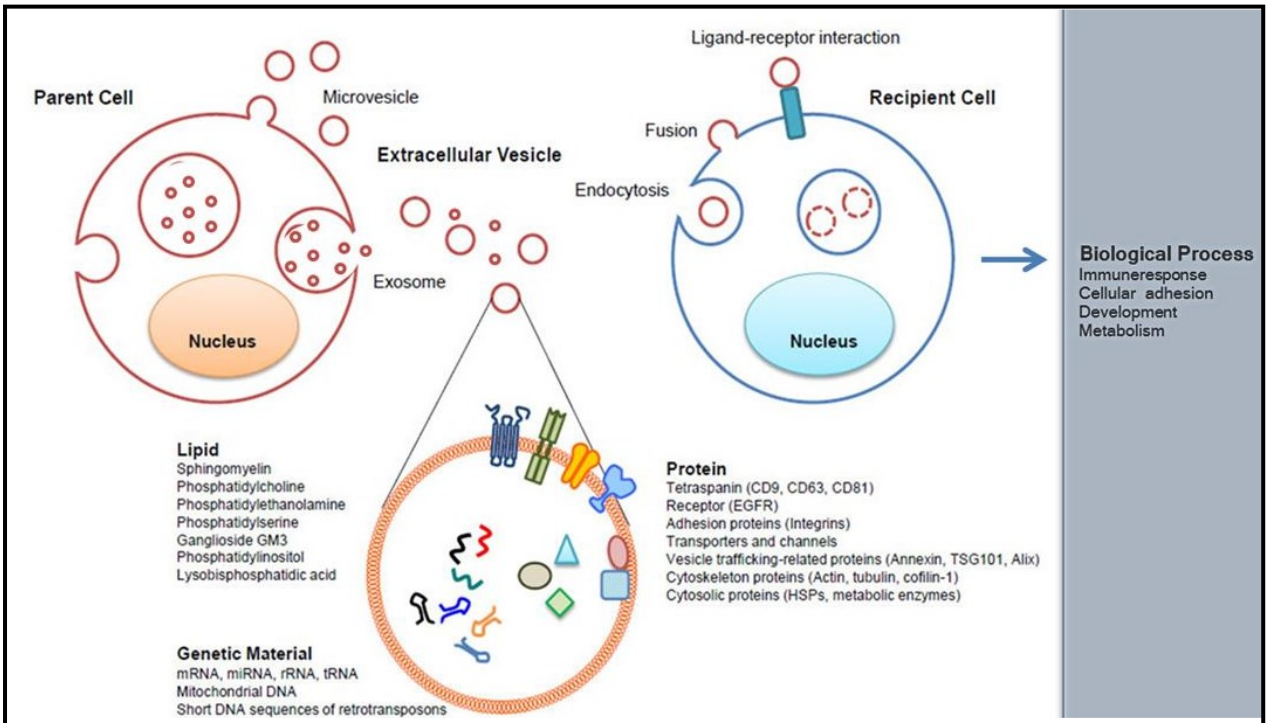


Figure 9. Intercellular communication via extracellular vesicles. EMVs are intercellular communicasomes harboring diverse bioactive materials including RNAs, DNAs, proteins, and lipids. EMVs regulate diverse range of pathophysiological functions by activating receptors of transferring membrane proteins, signaling molecules, mRNAs, and miRNAs. These EMVs can interact with the recipient cells by ligand-receptor interaction, fusion, and internalization via receptor-mediated endocytosis or macropinocytosis. EMVs secreted from the placenta can participate in an adaptive response of the mother and fetus and so interact with target tissue and modulate different biological processes, such as immune response, cellular adhesion, development and metabolism. (Modified after [102]).

1.7. Pregnancy-related disease: Preeclampsia

Various investigations have demonstrated relevance of miRNAs expression profiles in clinicopathological phenotypes and their possible potential as biomarkers in diverse human disorders, such as cancer, cardiovascular diseases, neurological disorders, viral infections [103] and pregnancy pathologies associated with placenta and fetal complications [104].

It is already known that shallow trophoblast invasion and incomplete endovascular transformation are leading causes of pregnancy disorders including preeclampsia, gestational diabetes (GDM), intrauterine growth restriction (IUGR) and low birth weight (LBW) [67] .

Preeclampsia (PE) is one common multi-system obstetric disorder naturally occurring only in primates and humans [105]. Around 2% to 8% of all pregnancies are affected by this syndrome and it has been catalogued as one of the 3 leading causes of maternal and perinatal morbidity and mortality worldwide [106-108]. Additionally, up to 2% of women with PE will progress to eclampsia leading to convulsions and potential maternal and fetal death [109]. Preeclampsia is characterized by hypertension (≥140/90 mmHg on two occasions) and proteinuria (≥300 mg protein/day) after 20 weeks of gestation [110]. Depending on the week of pregnancy at which the PE occurs, it can be subdivided into mild (also denoted early) and severe (also denoted late), initiating before 34 and after 34 weeks of pregnancy, respectively [109].

This systematic disorder may also affect the liver and the brain in the mother [111] and is often associated to diverse pathological processes as endothelial dysfunction, impaired implantation, increased intravascular coagulation, microcirculation, fibrin deposition and systematic inflammation in the mother [106, 109, 110]. PE is often related to complications in the developing fetus including prematurity, oligohydramnios, bronchopulmonary dysplasia, intrauterine growth restriction (IUGR), which is also characterized by trophoblast hypoxia and defective placental development or function, and increasing risk of perinatal death [111, 112].

As mentioned above, during normal placentation, the embryo-derived cytotrophoblast cells invade the maternal uterine wall and induce the remodeling of maternal vessels into high capacitance, low resistance vessels which provide access to maternal oxygen and nutrients for the placenta and developing fetus [113]. As a result of this process, the cytotrophoblasts adopt an endothelial phenotype, expressing adhesion molecules classically found on the surface of endothelial cells. In PE this process is restricted, characterized by shallow cytotrophoblast invasion only in the superficial layers of the decidua without adopting an endothelial adhesion phenotype. Finally, the spiral arteries fail to be remodeled, resulting in constricted, high resistance vessels (**Fig. 10**) [111].

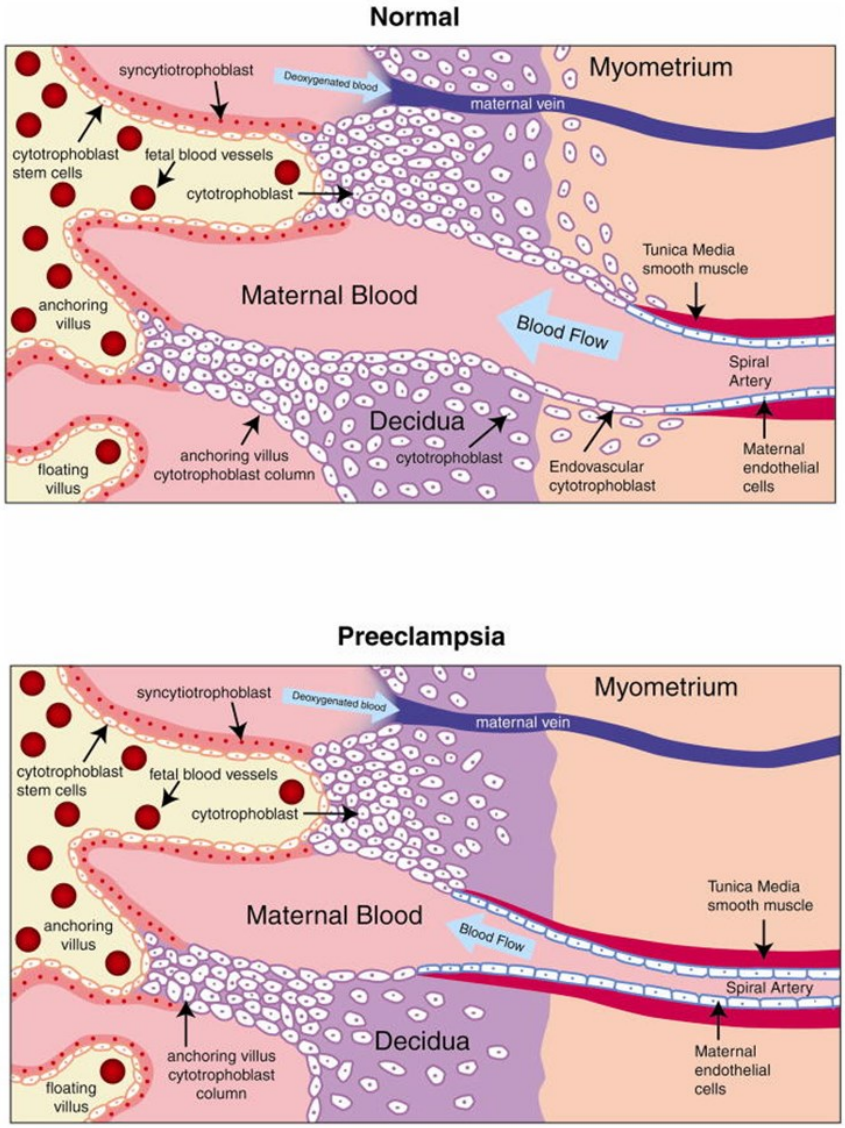


Figure 10. Abnormal Placentation in Preeclampsia. In normal placental development, invasive cytotrophoblasts of fetal origin invade the maternal spiral arteries, transforming them from small-caliber resistance vessels to high-caliber capacitance vessels capable of providing placental perfusion adequate to sustain the growing fetus. During the process of vascular invasion, the cytotrophoblasts differentiate from an epithelial phenotype to an endothelial phenotype, a process referred to as "pseudovasculogenesis" or "vascular mimicry" (Upper Panel). In preeclampsia, cytotrophoblasts fail to adopt an invasive endothelial phenotype. Instead, invasion of the spiral arteries is shallow and they remain small caliber, resistance vessels (Lower Panel) (Taken from [111]).

Since the past decade, the pathogenesis of preeclampsia has been a frequent topic of studies investigating the heterogeneous nature of this syndrome but its etiology remains unknown [110]. Several hypotheses have been postulated in an attempt to explain its pathogenesis. Some of them suggest that PE can be a result of failure in villous trophoblast

differentiation early in pregnancy, which leads to necrosis and apoptosis of trophoblast cells and increased release of fragments into the maternal circulation, such as EMVs [109].

Due to the incomplete knowledge on PE, effective preventions or treatments still have to be discovered. The most common solution is to deliver the baby and the placenta, often prematurely, in the interest of providing the most appropriate conditions for the newborns or their mothers [114]. Currently, it is assumed that a better understanding of the role of EMVs containing miRNA in PE can contribute to a better comprehension of this etiopathogenesis and promising clinical approaches [109].

Recent studies have shown a large number of miRNAs with potential importance in PE [67, 68, 115]. Several, indicated a relation between miRNAs and exosomes or MVs and its different roles in normal pregnancies and in PE. However, more research for tracking the trafficking of EMVs and miRNA cargo is required. The elucidation of this topic will be important for optimization of tools for therapeutic targeting of miRNAs in this and other disorders.

Chapter 2/Aims

2.1. General objectives

To elucidate molecular mechanisms associated with EGF signaling and miRNA gene regulation which may be responsible for the control of trophoblast proliferation and invasion in normal and preeclamptic pregnancies.

To contribute to the identification of novel miRNAs as potential diagnostic tools for therapeutic targeting of pregnancy disorders.

2.2. Specific objectives:

To investigate the function of EGF on trophoblast cell proliferation and invasion through activation of STAT5.

To analyze the role of miR-141 and miR-134 in the regulation of central functions of trophoblast cells: proliferation and invasion.

To compare the expression of miR-141 in normal and preeclamptic placentas and to investigate its role in the intercellular communication mediated by microvesicles.

This study will be subdivided in three parts:

- Analysis of EGF functions in trophoblast models (**Fig. 11A**).
- Investigation of effects of miR-141 and miR-134 on the regulation of trophoblast cell lines (**Fig. 11B**).
- Analysis of miR-141 expression in normal and preeclamptic placentas and of its role in the intracellular communication mediated by microvesicles (**Fig. 11C**).

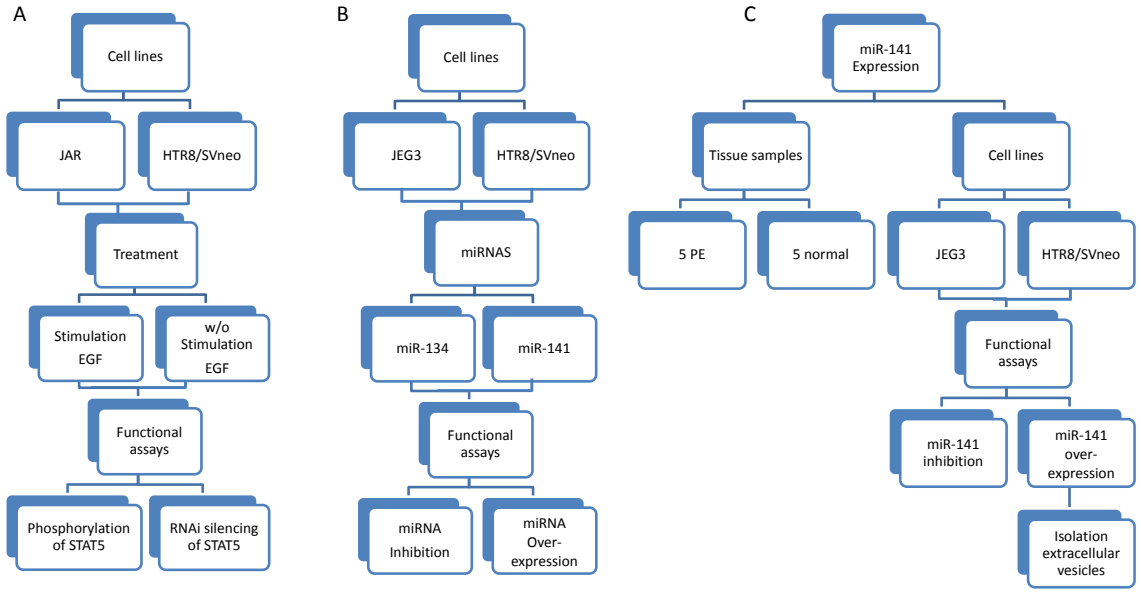


Figure 11. Experimental design of this work. **A.** Function of EGF in trophoblastic cells through regulation of STAT5 activation. **B.** MiRNAs expression in trophoblastic cell lines. **C.** MiR-141 expression in normal and preeclamptic placentas.

Chapter 3 / Materials and Methods

3.1. Human cell lines and cell culture

The trophoblastic cell lines JEG-3 and JAR were commercially obtained from ATCC (USA, Cat-No. HTB-36 or HTB-144). HTR-8/SVneo was obtained as a kind gift from Professor Charles Graham of the Department of Anatomy and Cell Biology at Queen's University, Kingston, ON, Canada. All cultures were commenced at 10^6 cells/175 cm^2 flask and cultured in F12 or RPMI-1640 medium (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 10% fetal bovine serum (FBS), and 1% of penicillin and streptomycin (PAA Laboratories GmbH) under standard conditions of 5% CO2 at 37°C and medium renewal every 2 days. Consequently, Jurkat, a human T cell line, (DSMZ, Braunschweig, Germany) was cultured in RMPI-1640 medium (PAA Laboratories GmbH, Cölbe, Germany) under the same conditions as applied for the trophoblastic cell lines. The cells were trypsinized regularly and adjusted to 10^5 cells/ml. The cell lines were inspected for absence of mycoplasma on a routine basis.

Extravillous trophoblast cells were isolated from term placenta tissue using a modified Kliman method as described in detail by Stenqvist [116] and standardized in Placenta Labor. Placental tissue was physically disaggregated and enzymatically digested for 30 min. After washing, isolated cells were applied on a density gradient (Percoll, Pharmacia, Sweden) and the fraction retained within the layer of 25% Percoll was collected and washed. For depletion of non-trophoblastic cells, Dynabeads coated with anti-CD45 and anti-CD82 antibodies (Life Technologies, Darmstadt, Germany) were used. Before use for RNA isolation, isolated cells were cultured for two days in F-12 medium (PAA) supplemented with 10% FCS. Adherent cells were checked for purity by flow cytometry using anti-EGF-receptor, anti-cytokeratin-7, and anti-HLA-G antibodies.

3.1.1. JAR and JEG-3 cell lines

The JAR cell line has been established by R. A. Pattillo and derives directly from a placental trophoblastic tumor of a 24-years-old caucasian woman (Patillo and Grey 1968). The JEG-3 cell line was established by Kohler in the early 1970's from a human gestational

choriocarcinoma that was taken at an autopsy from a cerebral metastasis (Kohler and Bridson 1971) (**Fig. 12**).

JAR and JEG-3 cells grow in vitro as monolayers and exhibit epithelial morphology [117]. Some dissimilarities between JEG-3 cells and villous trophoblast phenotypes have been reported, including polyploidy and expression of membrane bound HLA-E and HLA-C and soluble HLA-G1 by JEG-3 cells [118]. Despite them, JEG-3 cells are widely used as model for trophoblast behavior and several studies in JAR and JEG-3 cells could be extrapolated to trophoblast in vivo.

3.1.2. HTR-8/SVneo cell line

The immortalized human first-trimester trophoblast cell line HTR-8/SV40 was established in 1993 by Lala PK and associated. They introduced the gene encoding simian virus 40 large T antigens into first trimester human extravillous trophoblast cells. The resulting cell line shares several phenotypic properties with the parental trophoblasts but with an extension of the lifespan [119] (**Fig. 12**).

3.1.3. Jurkat cell line

The Jurkat cell line is an immortalized line of human T lymphocyte cells obtained for screening purposes from John Hansen at the Fred Hutchinson Cancer Research Center, Seattle, USA. However, during its standardization, the cells were heavily contaminated with mycoplasma. The process of curing the cell line of this infection yielded the Jurkat E6-1 clone, which eventually became the standard Jurkat cell line used by many T cell immunologists. This human T cell line spontaneously or inducibly releases large quantities of IL-2 after stimulation with phytohaemagglutinin (PHA) [120] (**Fig. 12**).

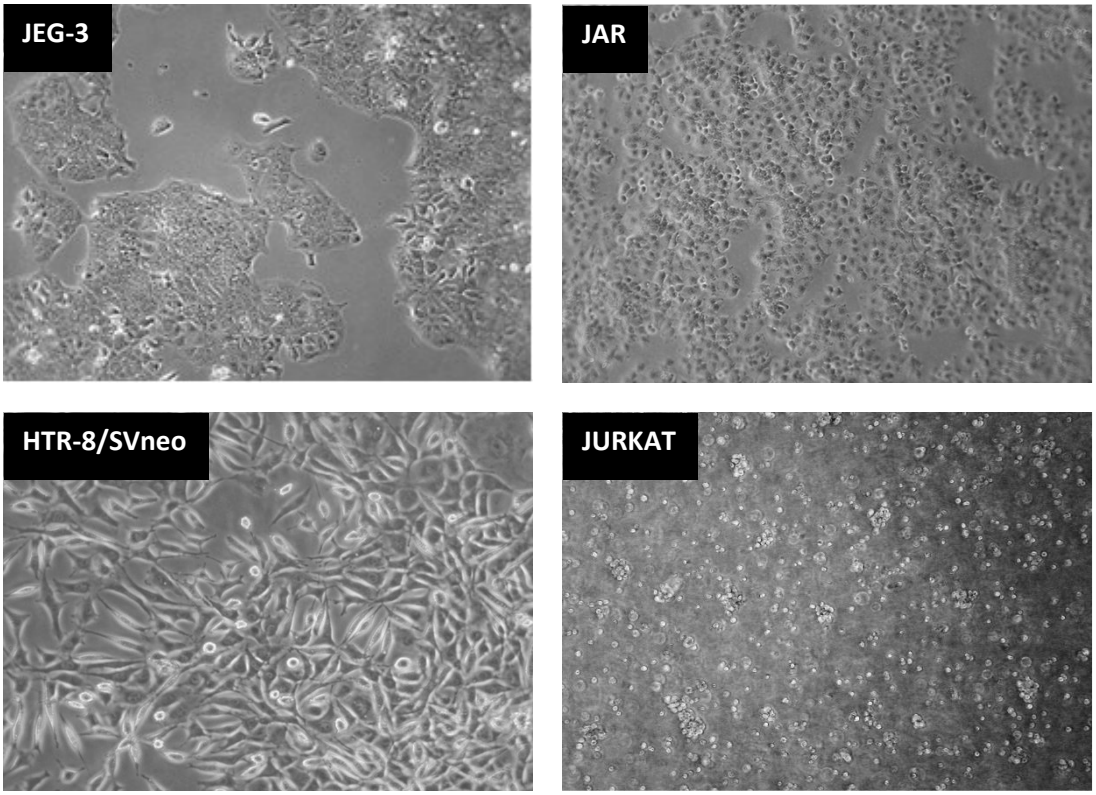


Figure 12. Human cell lines used for this thesis. The trophoblastic cell lines JEG-3 and JAR, the immortalized human trophoblast cell line HTR-8/SVneo (adherent cell lines) and human T lymphocyte cell line Jurkat (suspension cell line).

3.2. Cell Stimulation

All cell lines were treated with cytokines and growth factors at various concentrations. EGF (100 ng/ml) and LIF (10 ng/ml) were purchased from Millipore (Bellerica, MA, USA). GM-CSF (20 ng/ml); IL-11 (20 ng/ml) and IL-2 (20 ng/ml) were purchased from Immunotools (Friesoythe, Germany).

3.3. STAT5 mRNA expression in trophoblastic cells

Extravillous trophoblast cells were isolated from term placenta tissue and cultured for two days before of RNA isolation. Briefly, JAR and HTR-8/SVneo cells were seeded in 6-well plates and serum deprived for 2 h before total RNA extraction with Trizol reagent (Invitrogen, Darmstadt, Germany). Total RNA concentration and quality were determined at a NanoDrop

ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Samples with A260/A280 ratio greater than 1.8 were stored at −80°C until being processed.

50 ng of total RNA were reverse-transcribed using High capacity RNA-to-cDNA kit (Applied Biosystems Darmstadt, Germany). Real-time qPCR was performed using TaqMan gene expression assays with specific primers for STAT5B (Assay ID Hs00560035_m1), GAPDH (Hs03929097_g1) (Applied Biosystem, Darmstadt, Germany). Reactions were run in duplicates, including no-template controls in 96-well plates on an Mx3005P QPCR System (Applied Biosystems). Relative STAT5B mRNA expression was quantified by using the 2-DCt method relative to GAPDH.

3.4. Western blot assay

Cells were seeded in 6-well plates at a density of 1×10^5 cells and allowed to attach overnight. The next morning, cells were serum starved for 2 h before stimulation with EGF (100 ng/ml), GM-CSF (20 ng/ml), LIF (10 ng/ml), IL-11 (20 ng/ml) and IL-2 (20 ng/ml) for 15 min. After treatment, cells were washed with PBS and protein lysates were subjected in lysis buffer to three freeze/thaw cycles. Cell lysates were cleared from particles by centrifugation at 18,000 rpm for 30 min at 4°C. Protein concentration was measured using Bradford dye and a microplate reader according to the manufacturer´s protocol (Bio-Rad, Hercules, Calif, USA). For each sample, 20 µg of protein was re-suspended in 4% stacking gel (30% AB; 0.5 M SGP; 20% SDS; ddH2O; 20% APS and TEMED) and separated by 7.5% running gel (30% AB; 1.5 M TGP; 20% SDS; ddH2O; 20% APS and TEMED). Proteins were transferred to a PVDF membrane by electrophoretic transfer (Thermo Scientific, Rockford, IL, USA). Membranes were blocked with TBS-T (Tris-buffered saline containing Tween-20) containing 5% milk, blotted with rabbit primary antibody (1:1000) against phospho(p)-STAT5(tyr694), STAT5, p-STAT3(tyr705), STAT3, p-ERK1/2(thr202/tyr204), ERK1/2 or alpha-tubulin (all Cell Signaling, USA), overnight at 4°C. Blots were washed 3 times in TBS-T, incubated for 60 min with anti-rabbit IgG, HRP-linked antibody (Cell Signaling, USA), and washed with TBS-T. A chemiluminescent detection system (LuminataTM Forte, Millipore, US) was used and chemiluminescense of bands was analyzed on a ChemiBIS bio-imaging system (Biostep GmbH, Jahnsdorf, Germany).

3.5. STAT5 siRNA transfection

JAR and HTR-8/SVneo cells were seeded in 6-well plates at a density of 3×10^5 cells/well. After 12 h, cells were washed with OPTIMEM (GIBCO) and resuspended in 800 µl fresh OPTIMEM. Thereafter, cells were transfected with predesigned small interfering RNA (siRNA) for STAT5B or the control (Ambion). The following 5`-3` oligonucleotide sequence has been used: Sense: GCAUACACCAUUGCUUGGAAtt. The non-genomic control sequence was: GCCACUUAUAAAUUCGUUCtt. The transfection was performed with Oligofectamine Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer´s recommendations. Concentrations of oligonucleotides and Oligofectamine dilution were 66 nM and 1:2.75, respectively. After 4 h of treatment, transfections were stopped by addition of DMEM medium (Life technologies) containing 30% FBS. Cells were incubated at 37°C overnight before seeding for viability, proliferation and invasion assays.

3.6. Immunocytochemistry

JAR and HTR-8/SVneo cells were seeded at a density of 1.5×10^4 cells on Microscope Slides (Thermo Scientific™), allowing them to attach overnight, and then stimulated or not with EGF (100 ng/ml) for 15 min. Staining of cells was performed by using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, USA) as follows: cells were fixed in ethanol/methanol 1:1 for 5 min, washed in 0.1 M PBS and nonspecific antigens were blocked with normal goat serum for 20 min at RT. After blocking, slides were incubated 1 h with the primary antibody diluted 1:100 (anti-p-STAT5) (Cell Signaling) or Rabbit (DA1E) mAb IgG Isotype Control (Cell Signaling) in Antibody Diluent (DAKO, Hamburg, Germany), rinsed thrice and incubated 30 min with biotinylated affinity-purified anti-rabbit-IgG (Cell Signaling). Thereafter, slides were treated with a solution of Avidin/Biotinylated enzyme Complex (ABC; Thermo Fisher Scientific, Bonn, Germany) for 30 min, followed by 2 min staining with NovaRed-Kit (Vector Laboratories; Burlingame, CA, USA), and counterstained with hematoxylin for 2 min. Finally, slides were dehydrated by an ethanol-to-xylene treatment and covered with Histofluid (Paul Marienfeld, Lauda-Königshofen, Germany). Staining images were visualized on a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany).

3.7. DNA-Binding capability assay

Cells were cultured in 6-well plates at a density of 1×10^5 cells and stimulated with EGF (100 ng/ml) for 30 min. Nuclear and cytoplasmic protein extraction was performed using a Nuclear Extract Kit (Active Motif, Belgium) according to the manufacturer's instructions. Protein concentrations were assessed by a Bradford assay (Bio-Rad, Hercules, CA, USA). DNA-binding detection was performed by using a Transcription Factor Assay Kit (Active Motif, Belgium). Briefly, nuclear proteins were incubated on an oligonucleotide coated plate and incubated with a 1:1000 dilution of anti-STAT5 antibody in 1x antibody binding buffer for 1 h. The secondary HRP-conjugated antibody (1:1000) was applied for 1 h. Nb2 (prolactin stimulated) was used as a positive control. Developing solution was added for 10 min at RT followed by addition of stop solution. Absorbance was read in a SPECTROstar Omega (BMG Labtech, Offenburg, Germany) at 450 nm with a reference wavelength of 655 nm.

3.8. Functional Assays

3.8.1. Cell Viability

JAR and HTR-8/SVneo cells (10000 cells/well) were seeded in 96-well plates, allowing them to attach overnight and then serum starved for 2 h before stimulation with 100 ng/ml EGF. Cell viability was analyzed with a tetrazolium salt (MTS) assay (CellTiter96™, Promega, USA) after 0 (non-stimulated), 24 and 48 h stimulation. Briefly, new RPMI medium containing MTS (1:6 dilution) was added to the cells and color development was measured as optical density (OD) at 490 nm on a ELISA plate reader (SPECTROstar Omega; BMG Labtech, Offenburg, Germany). The color reaction of this assay is dependent on tetrazolium dye reduction through NAD(P)H-dependent oxidoreductase enzymes which correlates with the cellular metabolic activity. Cell viability at all time points was expressed as blank-corrected OD normalized to that of non-treated cells at 0 h.

3.8.2. Proliferation assay

JAR and HTR-8/SVneo cells (10000 cells/well) were seeded in 96-well plates, allowed to attach overnight and then serum starved for 2 h before stimulation with 100 ng/ml EGF. Cell proliferation was assessed by "Cell proliferation ELISA, BrdU" assay (Roche, Germany) at 0 (non-stimulated), 24 and 48 h stimulation. After treatment, cells were incubated 2 h with new RPMI containing BrdU labeling solution (1:1000). After removing the medium, cells were fixed and the DNA was denatured by treatment with FixDenat. Then, wells were incubated 90 min with peroxidase labelled anti-BrdU-Fab fragments. After rinsing, the immune complexes were detected by incubation with tetramethyl-benzidine (TMB). After 30 min, color development was measured at 370 and 492 nm on an ELISA plate reader (SPECTROstar Omega; BMG Labtech, Offenburg, Germany). Corrected absorbance values (A_{370}-A_{492}) correlate with the amount of DNA synthesis and thus, with the number of proliferating cells. Cell proliferation at all-time points was expressed as corrected absorbance and normalized to that of non-treated cells at 0 h.

3.8.3. Cell Invasion assay

Invasion assays were performed in 24-well plates by using transwell inserts of 8 µm pore size (BD Biosciences) coated with 1:3 diluted Matrigel (BD Biosciences). 1 x 10^5 cells per well were placed on the upper side of the inserts in 200 µl of medium containing or not EGF (100 ng/ml). After 24 h, cells in the lower side of the inserts were fixed in cold ethanol and stained with 0.1 % crystal violet. Formed color crystals were resolved in acetic acid and solutions were transferred into 96-well plates. Absorbance was measured at 570 nm in a SPECTROstar Omega plate reader (BMG Labtech). Cell invasion was expressed as absorbance of treated cells normalized to that of controls.

3.9. Patient samples

Patients aged between 18 – 32 years were recruited for the analysis from the University Hospital Jena, Germany. Five patients with confirmed diagnosis of preeclampsia and five age-matched controls were included. Tissues of the placental villi were collected

immediately after delivery under visual observation and washed with physiological serum to remove excess blood cells. Samples were placed into cryotubes, covered completely with RNA later (Ambion, Austin, TX, USA) and stored at -80°C until RNA extraction. All samples were collected with the informed consent of the patients. The project was approved by the Ethics Committee of University Hospital Jena.

3.10. RNA Isolation and Quantitative Real-time PCR

JEG3 and HTR-8/SVneo cells were seeded in 6-well plates and serum deprived for 2 h and total RNA was extracted from cells using Trizol reagent as per the manufacturer´s instructions (Invitrogen, Darmstadt, Germany). Total RNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Samples with A260/A280 ratio greater than 1.8 were stored at −80°C until being processed.

Blocks of 1 cm diameter of each placenta were cut with a mean weight of ~ 100 mg. Thereafter, one of the samples was transferred to a disposable for biological sample disaggregation (Medicons) for use on a Medimachine according to the manufacturer's protocol (C.T.S.V., Brescia, Italy). The cellular suspension was collected and subsequently, total RNA was isolated by using a mirVana isolation kit (Life Technologies), according to the manufacturer's protocol.

Thereafter, 100 ng or 10 ng of total RNA, obtained from cell lines or tissues samples, respectively, were reverse-transcribed, and real-time PCR was performed using TaqMan miRNA assays with specific primers for hsa-miR-141 (UAACACUGUCUGGUAAAGAUGG), and the endogenous control, RNU48 (GATGACCCCAGGTAACTCTGAGTGTGTCGCTGA TGCCATCACCGCAGCGCTCTGACC), (Applied Biosystem, Darmstadt, Germany). All reactions were run in duplicates, including no-template controls in 96-well plates on an Mx3005P QPCR System (Applied Biosystems). The experiments from cell lines were repeated three times and Student´s t-test was performed ($p< 0.05$).

3.11. Transfection with miR-141 mimic and antagomir

Cells were seeded in 6-well plates allowed to attach overnight to reach 30-50% confluence at the time of transfection. Expression of miR-141 was analyzed with either MiRIDIAN microRNA Mimic (Pre-miR-141) or Hairpin inhibitor (Anti-miR-141) specifically designed for these miRNAs or the respective non-genomic negative controls (Thermo Fisher Scientific, Schwerte, Germany). These miRNA were transfected into both trophoblastic cell lines at a final concentration of 25 nM by using Oligofectamine reagent (Invitrogen life technologies, Darmstadt, Germany) according to the manufacturer´s recommendations. After 24 hours of transfection, cells were trypsinized and seeded into plates for viability, proliferation and invasion assay. Expression level of miR-141 was analyzed by real time qPCR. Transfection indicator (siGLO Green, Thermo Fisher Scientific, Schwerte, Germany) was used for monitoring transfection efficiency.

3.12. Isolation of microparticles or exosomes from cell lines' supernatants

After transfection with miR-141 mimic (Pre-miR-141) or the respective control, five ml of new medium supplemented with 10% FBS was added to the cells. After 2 days of culture, the supernatants of each treatment with MiRIDIAN were collected and transferred into new tubes. Thereafter, the samples were stored at -80°C until microparticles (MPs) or exosomes (EXOs) were extracted. After thawing of supernatants, the suspension was centrifuged twice at different speed in order to remove dead cells and cell debris. The obtained supernatants were centrifuged for 30 min at 18.890xg at 4°C and pellets containing the microparticles were separated from the supernatant. The supernatants were transferred into centrifuge tubes (Beckman Coulter) and the pellets (microparticles) were resuspended in 5 ml PBS and again centrifuged for 30 min at 18.890xg at 4°C for discarding contaminating proteins. The so generated pellets contained exclusively microparticles at reduced concentrations of free proteins. The supernatants were filtrated using filters with 0.8/0.2 µm pore size (Pall Life Sciences) and centrifuged at 100.000xg for 70 min at 4°C. Finally, the pellets were suspended in 5 ml PBS and a new centrifuged at 100.000xg for 70 min to obtain pellets containing exosomes. Total RNA was extracted from both pellets (microparticles and exosomes) by using Trizol reagent as per the manufacturer´s instructions (Invitrogen) and 10 ng of total

RNA, was reverse-transcribed, and real-time PCR was performed using TaqMan miRNA assays with specific primers for hsa-miR-141 as mentioned before. Microparticles and exosomes pellets were re-suspended in 1 ml PBS and stored at -80°C until being used in functional assays (**Fig. 13**).

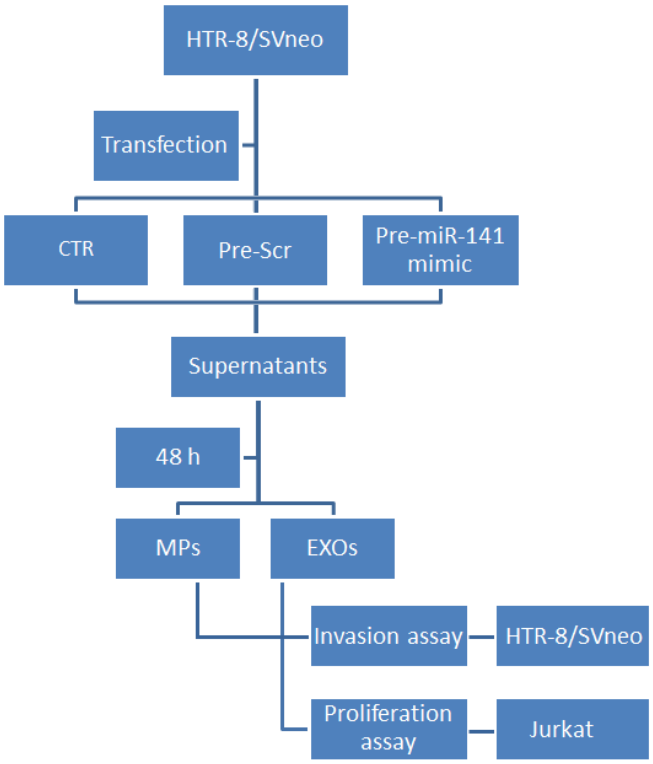


Figure 13. Experimental design for the isolation of microparticles (MPs) and exosomes (EXOs) from cell culture supernatants after over-expression of miR-141.

3.13. Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was performed using NanoSight Version 2.3 Build 0033 instrument (NanoSight Ltd., Amesbury, UK) following the manufacturer´s instructions. This instrument passes a focused 488 nm laser beam through a suspension of the sample of nanoparticles and collects the scattered light using conventional microscope optic aligned at 90° to the beam axis. Then, an electron multiplying charge coupled device captures a video of the field of view at 30 frames per second. The NanoSight instrument measures the rate of Brownian motion of each nanoparticle from frame to frame, thus enabling the calculation of

the hydrodynamic diameter via the Stokes-Einstein equation. Finally, the system provides a reproducible platform for specific and general nanoparticle characterization. Samples of MPs and EXOs were diluted in PBS over a range of concentrations to obtain between 10 and 100 particles per image prior to analysis with NTA system. Then, the respective solutions were introduced into the sample chamber and ten 20-second videos were recorded (shutter speed of 600; camera gain of 250). The captured videos were then processed and analyzed using optimized instrument settings (detection threshold 7 Multi; blur automatic; and Min Expected Size automatic) and an Excel spreadsheet (Microsoft Corp., Redmond, Washington) was automatically generated, showing mean, mode, and median particles size together with an estimate of the number of particles.

3.14. Statistics

All experiments were repeated at least three times. Statistical significance was performed using a two-tailed Student´s t-test. $p< 0.05$ was recognized as statistically significant.

Chapter 4/ Results

4.1. Function of EGF in trophoblastic cells through regulation of STAT5 activation.

First trimester extravillous cytotrophoblast (EVT) invasion plays an important role for placental growth and success of pregnancy. The process of placentation is regulated by cytokines and growth factors signaling via the JAK/STAT pathway. One of which is EGF.

Extravillous trophoblast cells were isolated from placenta tissue. STAT5B mRNA expression was similar in isolated trophoblast cells and HTR-8/SVneo cells but significantly lower in JAR cells when compared with primary cells (1.03-fold) (**Fig. 14**).

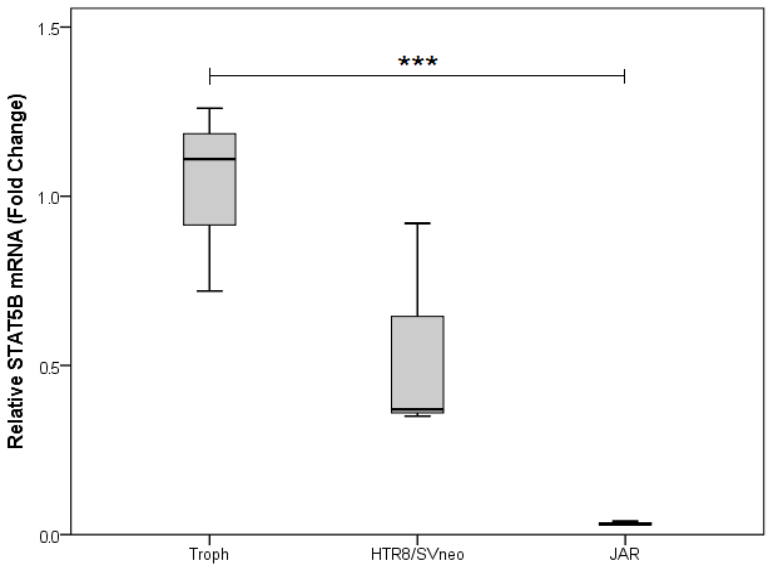


Figure 14. Basal STAT5B mRNA expression in isolated trophoblast cells and the cellular models HTR-8/SVneo and JAR cells. STAT5B mRNA levels are calculated as fold change ($2^{-\Delta\Delta Ct}$) with GAPDH as endogenous control and normalized to primary trophoblast cells (Troph). *** p< 0.005 (Student's t-test).

4.1.1. EGF induces STAT5 activation

Western blots demonstrate that only LIF (10 ng/ml) administration induces phosphorylation of p-STAT3 (tyr705) in both HTR-8/SVneo and JAR cells while p-STAT5 (tyr 694) is induced exclusively by EGF (100 ng/ml) (**Fig. 15A**). EGF-induced STAT5 phosphorylation is maximal

after 15 min and returns to background level after 30 min (data not shown). Basal expression of p-STAT5 is slightly higher in HTR-8/SVneo cells than in JAR cells as observed by Western blot and immunocytochemistry analysis (**Fig. 15A-B**). P-STAT5 was localized initially in the cytoplasm and after treatment with EGF, the levels in the nuclei increased (**Fig. 15B**).

To confirm that EGF induces STAT5 activation and translocation into the nucleus, DNA-binding capacity of STAT5 has been analyzed. It increases slightly, but not significantly upon stimulation with EGF in both cell lines (**Fig. 16**).

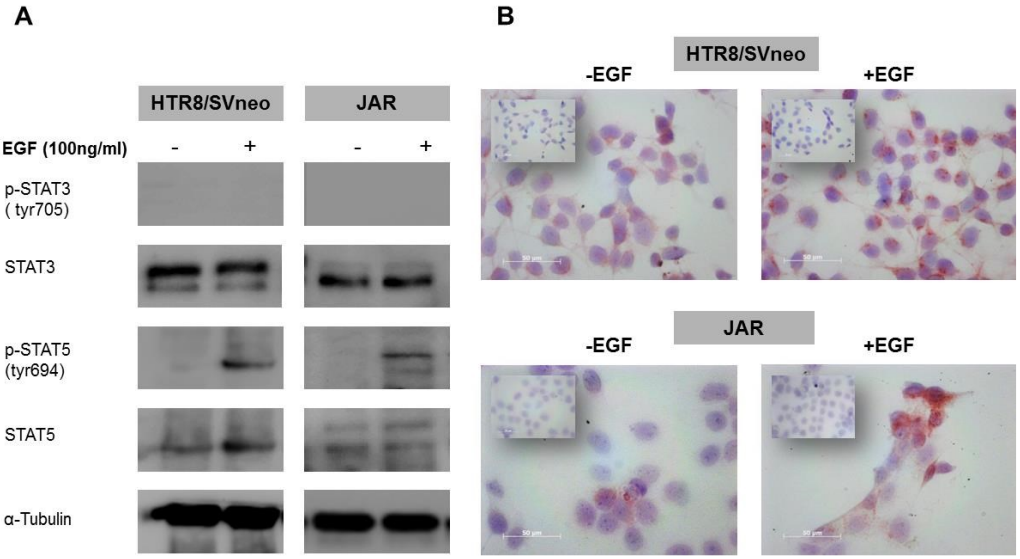


Figure 15. A. Effects of EGF on phosphorylation of STAT3 and STAT5 in HTR-8/SVneo and JAR cells. Representative Western blots of cells stimulated 15 min with EGF (100 ng/ml). B. Immunocytochemical staining of p-STAT5 (tyr694) in HTR-8/SVneo and JAR cells before (left panels) and after incubation (right panels) with EGF (100 ng/ml). Negative controls are shown in the upper corner of each treatment.

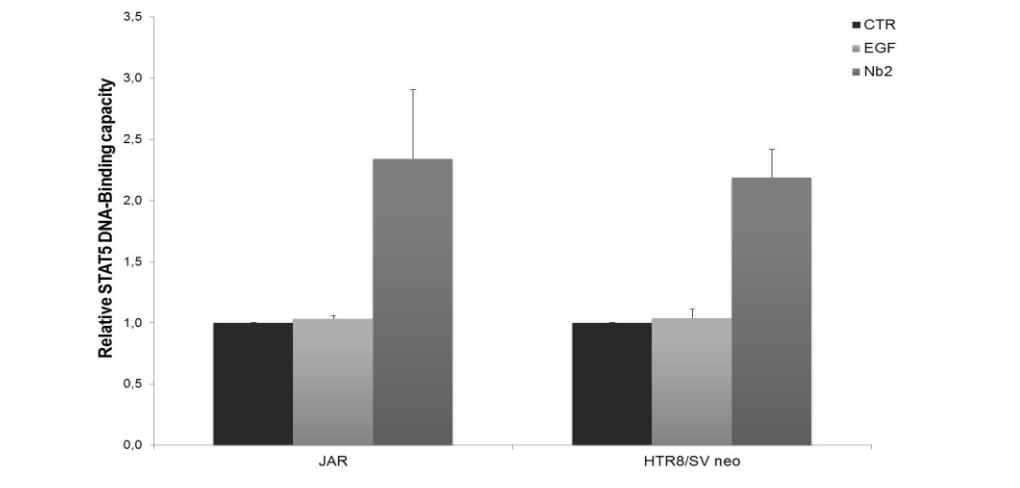


Figure 16. Relative STAT5 DNA-binding capacity of cells treated or not with EGF (100 ng/ml) for 30 min as assessed by a colorimetric assay. Values of controls have been defined as "1" and used for normalization. Bars represent mean values of n=3 independent experiments. Error bars indicate standard error of the mean. For positive control 5 µg lysate of the Nb2 lymphoma cell line has been used, which expresses constitutively phosphorylated STAT5.

4.1.2. EGF stimulates cell viability and invasion in a mechanism involving STAT5B

After 48h of treatment with EGF (100 ng/ml), viability of HTR-8/SVneo and JAR cells increases by 41% and 29%, respectively ($p < 0.05$; **Fig. 17A**). Cell proliferation assessed by BrdU incorporation was only increased in HTR-8/SVneo cells (by 14%) but not in JAR cells (**Fig. 17B**).

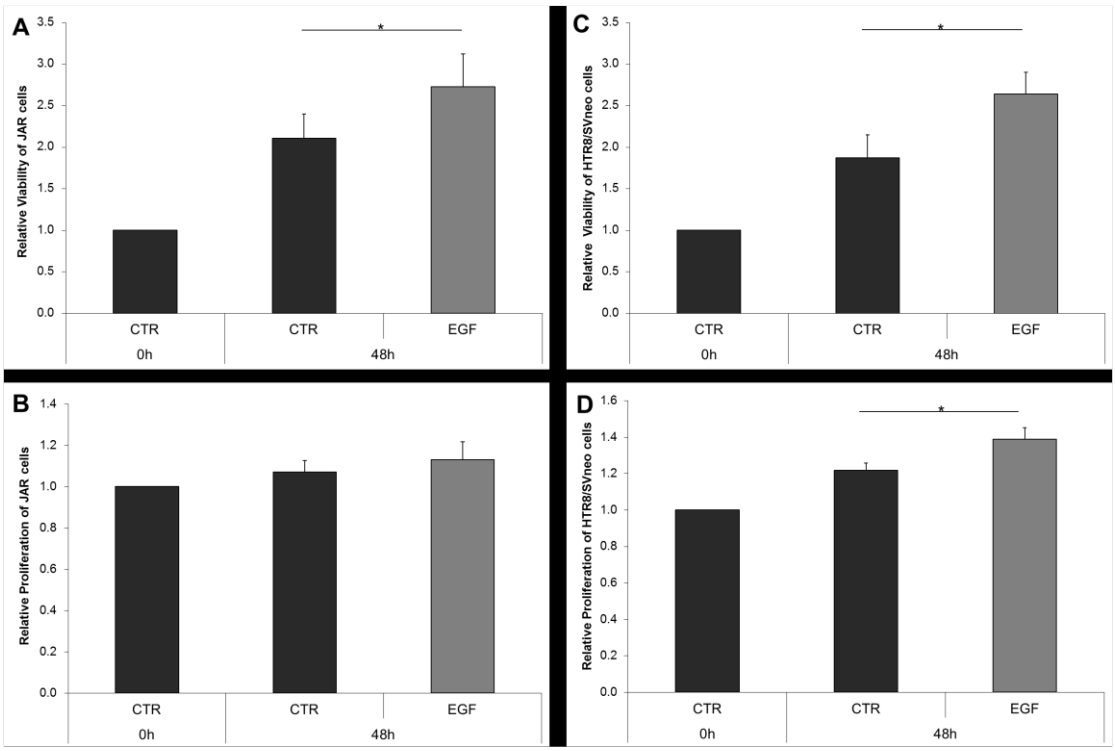


Figure 17. Effect of EGF on the viability of HTR-8/SVneo and JAR cells. Cells were incubated in presence or absence of EGF (100 ng/ml) for 48h. Cell viability and proliferation have been analyzed by MTS (A and C) or BrdU (B and D) colorimetric assays, respectively, as described in Material and Methods. Left panels: JAR cells, right panels: HTR-8/SVneo cells. Control values have been defined as "1" and used for normalization. Bars show mean values of n=4 independent experiments (each performed in triplicates) ± SEM. * $p < 0.05$ (Student´s t-test).

As confirmed by Western blotting, STAT5B was significantly down-regulated after transfection with STAT5B siRNA (Fig. 18). This knock-down results in a slight decrease of cell viability in both cell lines when compared to non-genomic transfected cells. In presence of EGF, cell viability of STAT5-silenced cells was significantly lower in comparison to that of controls (Fig. 18). Remarkably, cell viability in siSTAT5 transfected cells was similar before and after EGF treatment.

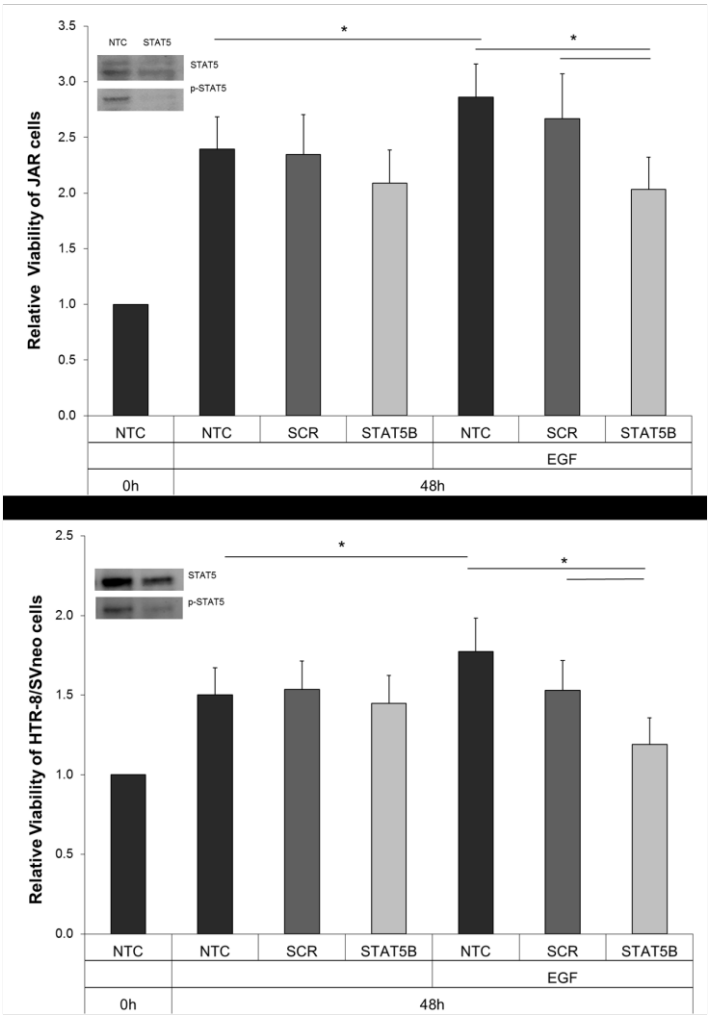


Figure 18. Relative viability of trophoblastic cells after STAT5B silencing and EGF stimulation. Cells were transfected with specific STAT5B siRNA or non-genomic siRNA and subsequently, incubated for up to 48 h in presence or absence of EGF (100 ng/ml). Results were normalized to non-stimulated cells at 0 h (non-stimulated cells, values defined as "1") and are expressed as mean (n=4 independent transfections, each experiment performed in triplicates). Error bars indicate SEM. (NTC: non-transfected cells, SCR: cells transfected with non-genomic siRNAs, STAT5B: siRNA specific for STAT5B. * $p < 0.05$ (Student's t-test) for the indicated comparison. Upper corners show representative Western blots of STAT5 expression before and after silencing.

4.1.3. EGF-enhanced HTR-8/SVneo invasion is mediated by STAT5

The capacity of cells to invade through Matrigel has been monitored as described in "Material and Methods". JAR cells show 25% higher spontaneous invasion than HTR-8/SVneo cells. Silencing of STAT5B alone did not affect invasion capability in any cell line. After 24h of treatment with EGF, invasion was increased in HTR-8/SVneo cells (1.35 fold) but not in JAR cells. EGF-stimulated invasion of HTR-8/SVneo cells was significantly reduced after STAT5 silencing by 34% (**Fig. 19**).

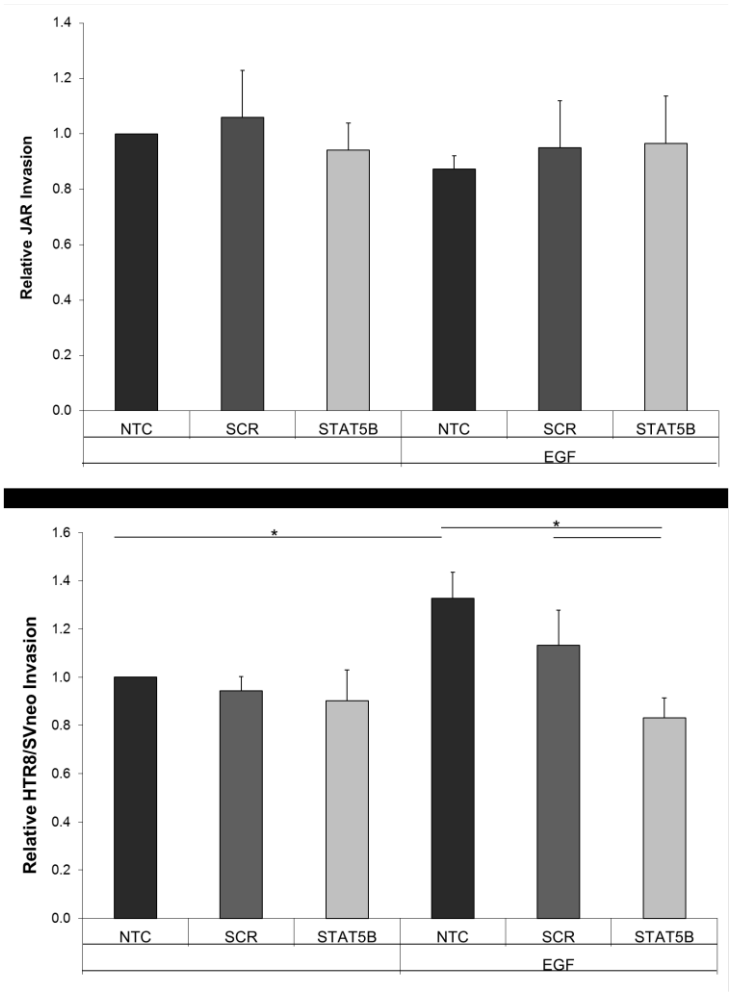


Figure 19. Relative invasiveness of trophoblastic cells after STAT5B silencing and EGF stimulation. Cells were transfected with STAT5 siRNA and seeded on transwell chambers coated with Matrigel in presence or absence of EGF (100 ng/ml). Relative invasion was measured after 24 h. Results are normalized to non-stimulated cells. Error bars indicate SEM. (NTC: non-transfected cells, SCR: cells transfected with scrambled, non-genomic siRNAs, STAT5B: siRNA specific for STAT5B. * $p < 0.05$ (Student's t-test) for the indicated comparison.

4.2. MiRNAs expression in trophoblastic cell lines and placenta tissue

4.2.1. Biological relevance of miRNAs in pregnancy

Human placenta tissue exhibits a specific miRNA expression pattern that dynamically changes with the gestational age and its alteration may result in pregnancy complications [68]. Two reviews providing a general overview on pregnancy-specific miRNAs were written. The first review showed the current knowledge on the origin and expression of human placenta miRNAs, as well as their interspecies differences [67]. In the second review, the state-of-the-art about the expression of pregnancy-related miRNA clusters (C14MC, C19MC and miR-371-3 cluster), their correlation with pregnancy disorders and their possible relevance as biomarkers for the detection and treatment of pregnancy-associated diseases were summarized [68].

Based on the information of the reviews and previous studies performed by our laboratory, the importance of expression of a larger miRNA clusters located on the chromosome 14 (C14MC) has been emphasized. Among the members of this cluster, miR-134 was highly expressed in first trimester but comparatively low in third trimester trophoblast cells [75]. Additionally, the literature and previous studies from our group report the importance of some miRNAs during pregnancy which are not localized within C14MC or C19MC. One of them is miR-141, a representative of the miR-200 family [73]. However, the role of miRNA-134 and miRNA-141 in trophoblast cells has not yet been investigated in detail.

In this part of the work, the expression of miR-134 and miR-141 were analyzed in the two trophoblastic cell lines JEG-3 and HTR-8/SVneo and their effects on proliferation and invasion, after inhibition or over-expression. Simultaneously, the expression of miR-141 in placenta tissues was analyzed (**Table 1**).

Table 1. Clinical characteristics of patients from which placenta tissues samples have been used in the study

	Normal		PE		p
	Average	SD	Average	SD	
Age, years	26.50	6.81	26.20	3.03	0.93
Gestational age, weeks	39.68	0.84	34.89	3.11	0.02
Birth weight, g	3520.00	140.65	2221.00	642.71	0.01
Mode of delivery: Cesarean section %	40.00		100.00		

4.2.2. Effect of miR-134-mimic or –antagomir transfection on cell proliferation and invasion

MiR-134 was highly expressed in HTR-8/SVneo but was lower or not expressed in JEG-3 cells (**Fig. 20**). Silencing of miR-134 in HTR-8/SVneo cells altered invasion when compared with non-genomic control, while overexpression did not changed (**Fig. 21 A-C**). Cell proliferation was significantly inhibited in HTR-8/SVneo cells transfected with miR-134-mimic but miR-134 inhibition did not lead to an increase (Fig. 21 B-D). Viability did not change significantly (data not show).

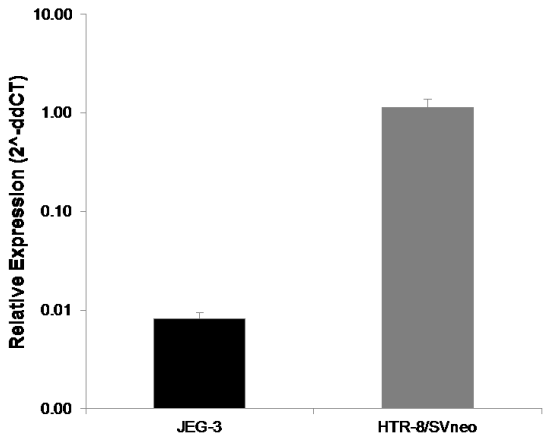


Figure 20. Basal miR-134 expression in JEG3 and HTR-8/SVneo cells. $2^{-\Delta\Delta Ct}$ was used to indicate miRNA expression relative to RNU48 (endogenous control) and normalized to HTR-8/SVneo cells. $\Delta\Delta Ct = (Ct_{miR\text{-}134} - Ct_{RNU48})_{Sample} - (Ct_{miR\text{-}134} - Ct_{RNU48})_{HTR\text{-}8/Svneo}$. Bars correspond to mean ± SD (n=4).

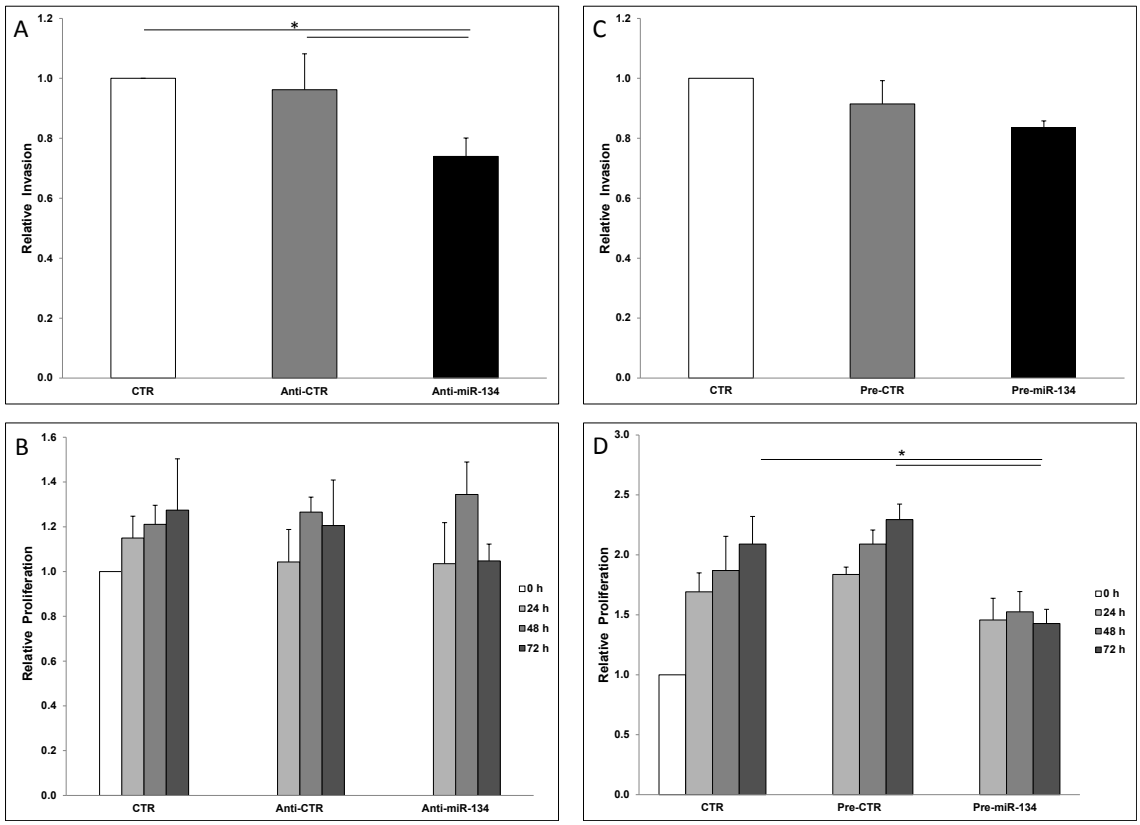


Figure 21. Effect of miR-134 inhibition or overexpression on invasion and proliferation of HTR-8/SVneo. Cells were transfected with either miR-134-antagonist (Anti-miR-134) or miR-134 mimic (Pre-miR-141) and invasion was assessed by Matrigel (A-B) or proliferation by BrdU (C-D) assays. CTR: non-transfected cells; Anti-CTR and Pre-CTR: non-genomic sequence, respectively to antagonist or mimic. * p< 0.05 (Student's t-test).

4.2.3. MiR-141 is upregulated in preeclamptic pregnancies tissues.

Expression levels of miR-141 were detected in 5 PE and 4 normal placental tissues samples by RT-qPCR. MiR-141 was significantly increased in PE placenta when compared with normal controls (1.64-fold $P < 0.05$). Basal levels of miR-141 were similar in JEG-3 and HTR-8/SVneo cells but were significantly lower than in normal tissues (**Fig. 22**). Transfection with miR-141-mimic resulted ~5,600-fold and ~3,993-fold increased expression in HTR-8/SVneo and JEG-3 cells, respectively. Conversely, treatment with miR-141-inhibitor reduced miR levels in 70% in HTR-8/SVneo and 60% in JEG-3 cells.

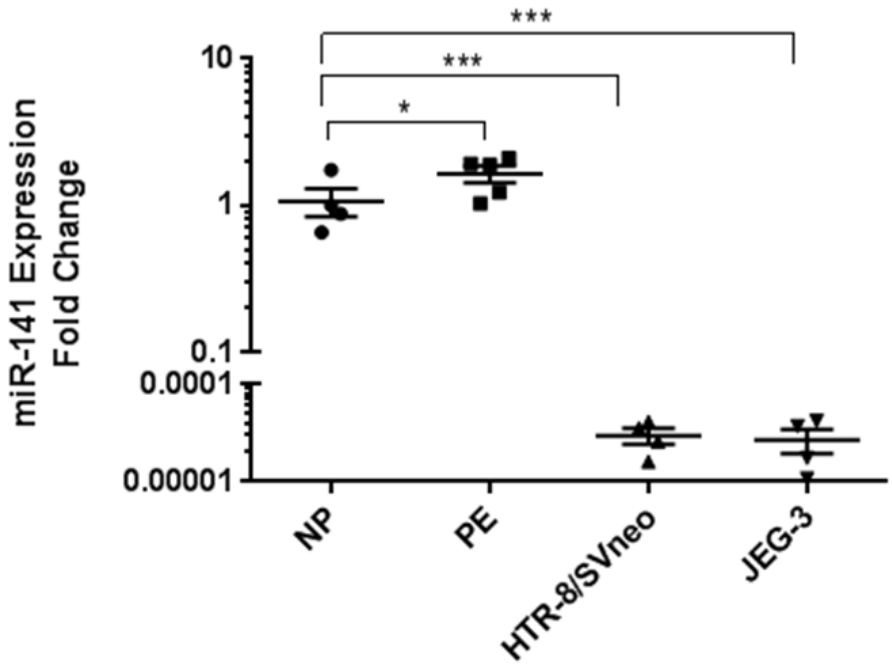


Figure 22. Scatter plot of miR-141 expression in placentas from preeclampsia (PE, n=5) and normal pregnancy (NP, n=4), or the trophoblast cell lines HTR-8/SVneo and JEG-3 (n=4). miR-141 expression was calculated as fold change (2-ΔΔCt) with RNU48 as endogenous control and normalized to NP. * p< 0.05 *** p< 0.001 (Student's t-test).

4.2.4. Effect of miR-141-mimic or –antagomir transfection on cell proliferation

Fourty-eight hours after transfection with neither miR-141-mimic nor miR-141-inhibitor No significant changes were observed in HTR-8/SVneo viability, measured by MTS assay, (**Fig. 23A-B**). After miR-141 inhibition, viability of JEG-3 cells decreased when compared with non-genomic control (p< 0.1; **Fig. 23A**) but miR-141 over-expression did not lead to an increase (**Fig. 23B**).

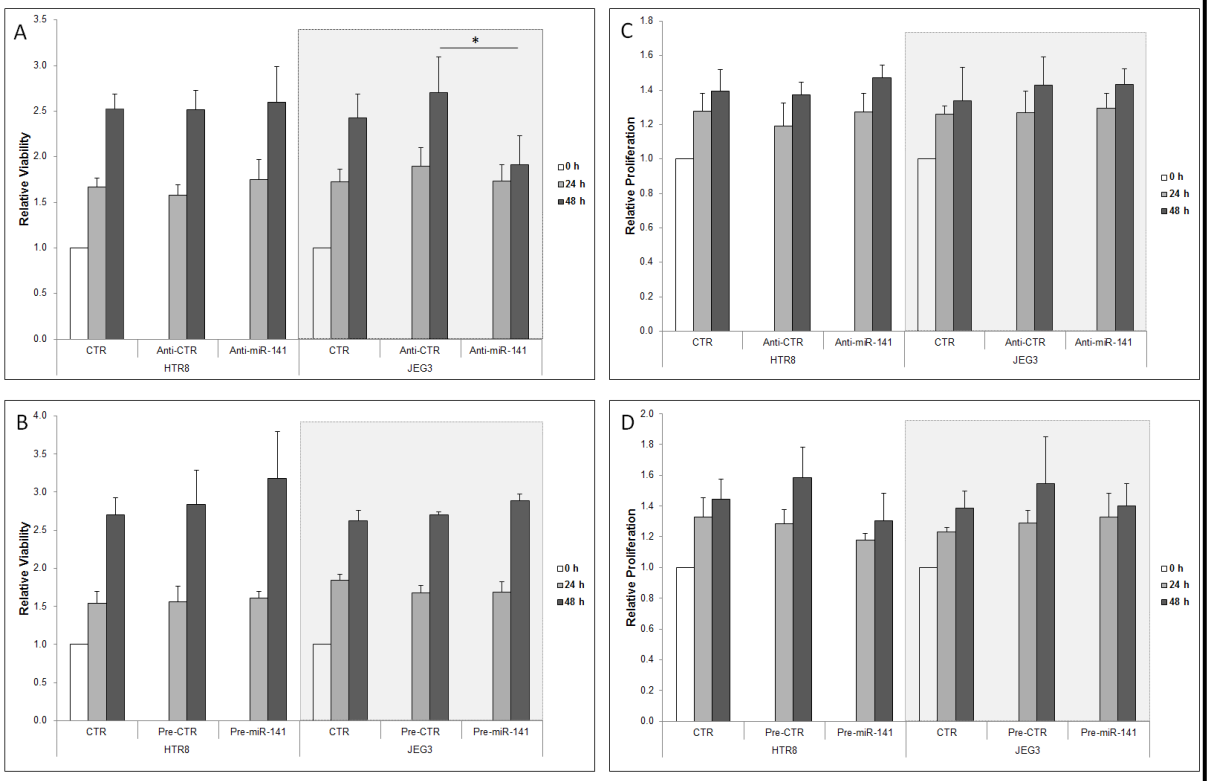


Figure 23. Effect of miR-141 inhibition or overexpression on viability and proliferation of trophoblastic cell lines. JEG-3 and HTR-8/SVneo cells were transfected with either miR-141-antagonist (Anti-miR-141; upper panel) or miR-141 mimic (Pre-miR-141; lower panel) and cell viability was assessed by MTS (a-b) or proliferation by BrdU (c-d) assays. CTR: non-transfected cells; Anti-CTR and Pre-CTR: non-genomic sequence, respectively to antagonist or mimic. * p< 0.05 (Student's t-test).

4.2.5. MiR-141 regulates cell invasion

The capacity of cells to invade through Matrigel has been monitored 48hr after inhibition or overexpression of miR-141. A direct correlation between miR-141 levels and cell invasion was confirmed in the HTR-8/SVneo cell line but not in JEG-3 cells. Cell invasion was significantly inhibited in both HTR-8/SVneo and JEG-3 cells transfected with anti-miR-141 (**Fig. 24A**), while it was significantly increased after transfection with miR-141 mimic only in HTR-8/SVneo cells (**Fig. 24B**).

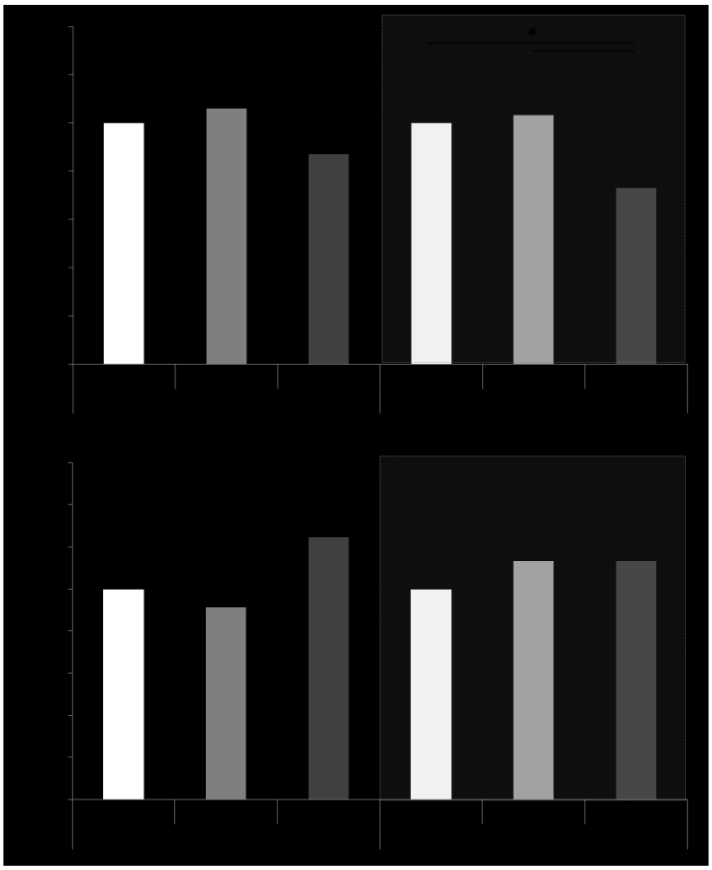


Figure 24. Effect of miR-141 inhibition or overexpression on invasiveness of trophoblastic cell lines. Cells were transfected with A. miR-141 inhibitor (Anti-miR-141) or B. miR-141 mimic (Pre-miR-141) or the respective controls (Anti-CTR and Pre-CTR) and invasiveness was assessed by Matrigel assay after 48 h. Bars represent mean ± SE. *p<0.05 (two-tailed Student's t test).

4.3. Characterization of isolated exosomes and microparticles

Microparticles (MPs) and exosomes (Exos) were isolated from JEG-3 and HTR-8/SVneo cell culture supertantants. Nanoparticle Tracking Analysis (NTA) showed spherical vesicles in both populations (**Fig. 25A** insert). Median size of particles in the MP-enriched fractions was 189 and 172 nm in JEG-3 and HTR-8/SVneo, respectively. Median size of particles in Exos-enriched fractions was 116 nm in JEG-3 and 112 nm in HTR-8/SVneo cells (**Fig. 25A**). Particle diameter distribution ranged respectively from 50-530 nm in microparticles and 30-500nm in exosomal fractions (**Fig. 25A**).

4.3.1. Assessment of miR-141 in microparticles and exosomes isolated from cell culture supernatants

Elevated miR-141 expression was observed in isolated microparticles and exosomes obtained from miR-141-mimic transfected cells when compared to those of non-transfected cells. Higher increase in miR-141 levels was detected in the microparticle fraction (~180-fold and ~120-fold increase, **Fig. 25B**) than in the exosomal fraction (~5.2-fold and ~5.3-fold increase, **Fig. 25B**) of transfected HTR-8/SVneo and JEG-3 cells, respectively. Levels of miR-141 in the microparticle fraction was 1.5-fold higher in JEG-3 cells than in HTR-8/SVneo (**Fig. 25B**) but similar expression was observed in the exosomal fraction of both cell lines (**Fig. 25B**).

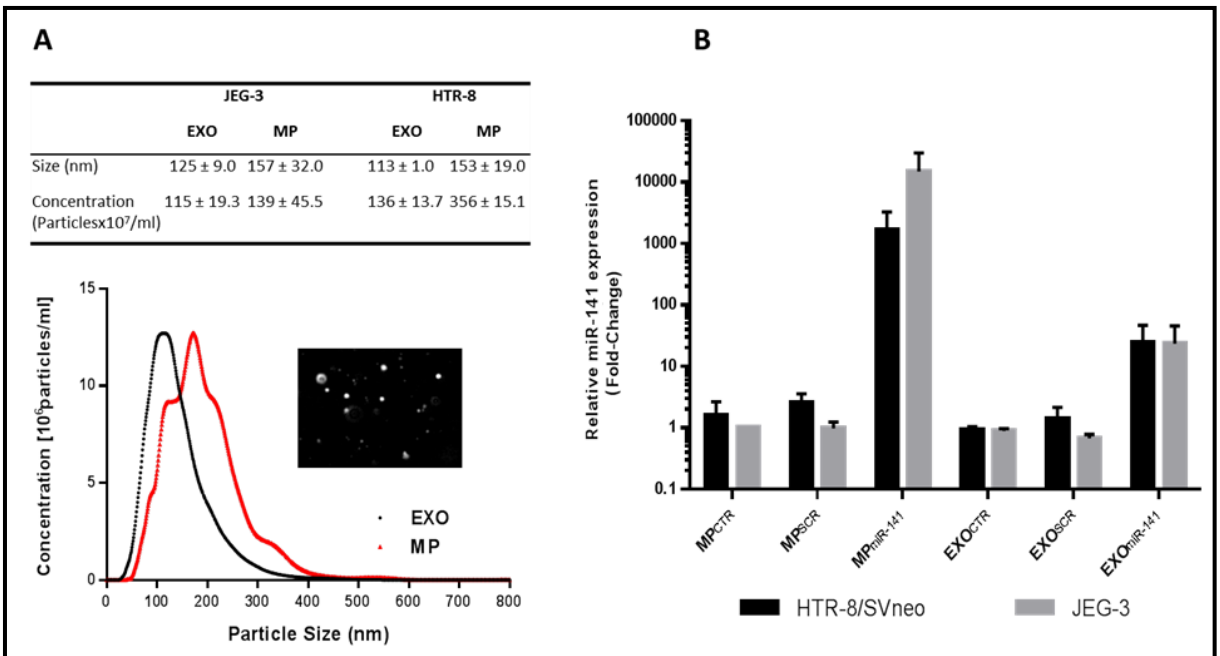


Figure 25. Characterization of two fractions of extracellular vesicles (EMVs) isolated from cell line supernatants. Based on different isolation protocols, the fractions were exosomes (EXO) and microparticles (MP) enriched. A. Particle size distribution and concentration of EXO and MP enriched fractions from JEG-3 and HTR-8/SVneo cell supernatants. Representative nanoparticle tracking analysis (NTA) for EXO (black line) and MP (red line) enriched fractions isolated from HTR-8/SVneo cell supernatants. Insert: Light scatter from isolated particles. B. qRT-PCR analysis of miR-141 expression in EMVs isolated from cell line supernatants before transfection (MP_{CTR} and EXO_{CTR}) and after transfection with scrambled sequences (MP_{SCR} and EXO_{SCR}) or miR-141-mimic ($MP_{miR-141}$ and $EXO_{miR-141}$). MiR-141 expression is expressed as fold change ±SEM with RNU48 as endogenous control and normalized to JEG-3 MP_{CTR}.

4.3.2. Trophoblastic cells do not auto-regulate invasion by secretion of miR-141-containing microvesicles

Non-transfected HTR-8-SVneo cells were seeded in a Matrigel assay chamber and then treated with isolated MPs or EXOs derived from cells which were non-transfected (CTR), transfected with miR-141 mimic (pre-miR-141) or with the respective non-genomic negative controls (pre-SCR). No significant changes were observed in the cell invasion capability of cells incubated with up to 100µl of any isolated MPs or EXOs (**Fig. 26A**).

4.3.2.1. Intercellular transfer of extracellular vesicles containing miR-141

Jurkat T cells were treated with increasing amounts of isolated MPs or EXOs from supernatans of non-transfected cells (CTR), cells transfected with miR-141 mimic (pre-miR-141) or with the respective non-genomic negative controls (pre-SCR). A significant dose-dependent reduction of Jurkat proliferation was observed after 48h of treatment with MPs and EXOs containing higher levels of miR-141 compared to that of basal levels. Treatment with high doses of EMVs reduced cell proliferation independently of the miR-141 content (**Fig. 26B**).

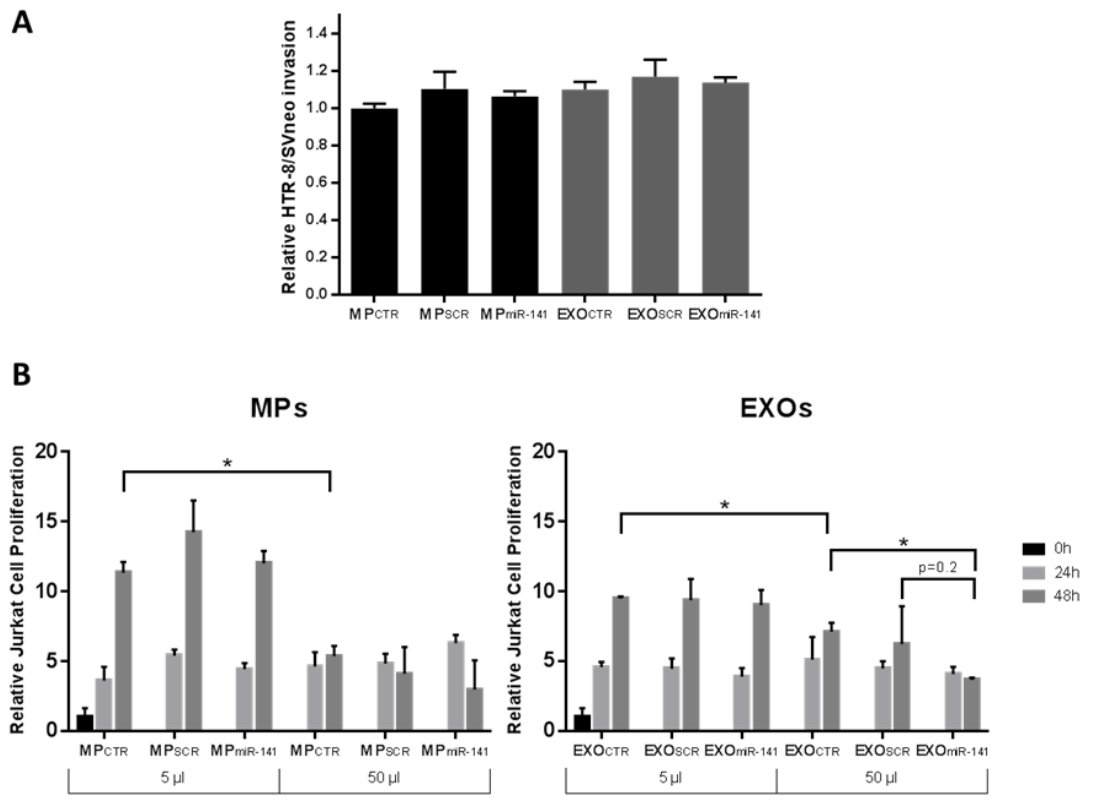


Figure 26. Intercellular communication mediated by microvesicles containing miR-141. Exosome- (EXO) and microparticle-enriched (MP) fractions were isolated from HTR-8/SVneo cell supernatants before (EXO_{CTR} and MP_{CTR}, respectively) and after transfection with scrambled non-genomic sequences (EXO_{SCR} and MP_{SCR}) or miR-141-mimic (MPmiR-141 and EXOmiR-141). **A.** Non-transfected HTR-8/SVneo cells were incubated with isolated microvesicles and relative invasion was assessed after 48h. **B.** After 24 h and 48 h treatment with isolated and differently treated MPs or EXOs, proliferation of Jurkat T cells was assessed by BrdU assays. All bars (in A and B) represent mean (n=3) ± SEM *p<0.05.

Chapter 5/ Discussion

5.1. EGF in trophoblast model.

Human pregnancy is considered a process where the mother´s immune system decides to tolerate and foster an incorporated, non-self, non-dangerous organism (tolerated semi-allogenic). The success of pregnancy depends on a bidirectional " feto-maternal dialogue", where the "messages" that are "spoken" are relayed through signaling mediators, which are capable of transmitting a functional command to a target cell [47]. Although there have been a number of studies considering the potential role(s) played by these mediators, cytokines or growth factors, at the time of implantation, its complex nature requires further clarity. Understanding the physiological processes in pregnancy enables effective management of pregnancy disorders threatening the mother and her fetus.

In the field of human reproduction, several investigations on different cell models have provided a large amount of information about different cytokines and growth factors and their association with pregnancy complications, such as preeclampsia and preterm delivery or recurrent miscarriage. Some are discussed in reviews, elucidating the important roles of EGF and LIF in trophoblast functions through a variety of intracellular pathways such as JAK/STAT and MAPK [47, 121, 122].

EGF is a pivotal growth factor, which influences a variety of fundamental cell properties needed for normal pregnancy [47]. The importance of EGF has been demonstrated *in vitro* and *in vivo. EGF* is associated with promoting growth of the developing fetus in pregnant women [122, 123].

EGF can activate STAT1, STAT3 or STAT5 either simultaneously or individually in a cell type-depended manner [29, 124, 125]. EGF treatment results in increase of only p-STAT3 in kidney tubular epithelial cells [126], but only p-STAT5 in U87 glioma cells [127]. In human breast cancer, STAT3 and STAT5 are activated simultaneously by EGF [125], while in squamous carcinoma cells, EGF activates STAT1 and STAT3 [128]. Previous studies in HTR-8/SVneo cells demonstrated that EGF triggers STAT5 phosphorylation but does not induce phosphorylation of either STAT1 or STAT3 [29]. This study goes in line with our results demonstrating activation of STAT5 but not of STAT3 after EGF treatment in HTR-8/SVneo

and JAR cells. These observations suggest that in trophoblast cells, EGF effects are mediated via STAT5 rather than via STAT3.

STATs are usually located in the cytoplasm but upon activation, they become phosphorylated, dimerize, and translocate into the nucleus where they bind to DNA and modulate gene expression [129]. Our results demonstrated high expression of STAT5B mRNA in isolated trophoblast cells which was similar to that of HTR8/SVneo but significantly lower in JAR cells. The protocol used in this study allows the isolation of extravillous trophoblast cells which are physiologically responsible for invasion during placenta formation [116]. The results highlight the importance of STAT5 during trophoblast invasion and demonstrate the suitability of the used cell lines models.

Using JAR and HTR-8/SVneo cells as models, we demonstrated that EGF effects are mediated via STAT5. The localization of p-STAT5 was in the nuclei of both cell lines after EGF treatment. These observations suggest activation of STAT5 after EGF stimulation, which was confirmed by Western blotting and immunocytochemistry. Not in line with these results, STAT5 DNA-binding activity was not altered by stimulation with EGF. We could measure only a low constitutive STAT5 DNA-binding activity. These results are congruent with previous observations demonstrating that some biological functions mediated by STAT5 are independent of its DNA binding and may be the result of consequent activation of other transcriptional factors [127], thus, the specific intracellular mechanism remains to be elucidated.

After confirmation of its EGF-induced activation, STAT5 silencing was conducted in both cell lines by using specific siRNA followed by EGF-stimulation. The expression of STAT5 was silenced by ~70%, as confirmed by Western blotting. Subsequently, viability, proliferation and invasion were analyzed.

The activation of different STATs depends on cell-type and stimuli. For this reason, upon EGF stimulation, cell functions vary between the different cell lines. In our study, EGF increases cell viability in both cell lines as assessed by MTS, and STAT5 silencing resulted in a decrease. However, JAR proliferation was not significantly altered after stimulation with EGF as assessed by BrdU assays and the effect in HTR-8/SVneo cells was lower than that observed by MTS assay. The MTS assay is often used as a proliferation test and thus, the dissimilarities

observed here may be due to differences in the sensibility and applicability of these assays. When STAT5 expression is silenced, the effect of EGF on cell viability is lost which demonstrates involvement of STAT5 in the increased cell viability induced by EGF. Other intracellular cascades like MAPK can also mediate EGF effects suggesting possible cross-talks that should be further investigated.

In the present work, enhanced invasiveness of HTR-8/SVneo cells after EGF treatment has been demonstrated. However, when STAT5 gene expression is silenced, EGF does not increase invasion although the STAT5 DNA-binding activity remains unaltered. This result is consistent with another study, where EGF enhances U87 glioma cell invasion via a mechanism independent of STAT5 DNA-binding [127].

In our hands, JAR cell invasion is not altered after EGF treatment. Previously, it was reported that EGF enhances invasion in JAR cells [130, 131]. This discrepancy may be explained by differences between methods. In our current study, nuclei of invasive cells have been stained with violet crystal after 24 h, while in the previous study, invaded cells were indirectly quantified by reduction of tetrazolium salts after 48-72 h [131].

Altogether, our data suggest that the intracellular mechanisms controlling invasion induced by EGF differ between JAR and HTR-8/SVneo cells. These results can be explained, as mentioned before, by the different origin of cell lines: JAR is a choriocarcinoma cell line while HTR-8/SVneo is a transformed cell line from first trimester trophoblast cells [132]. Also other investigations described major differences between HTR-8/SVneo and choriocarcinoma cell lines including dissimilarities in their protein and gene expression profiling [75, 133, 134]. Further studies are necessary for understanding the mechanisms of invasion in the different cell lines.

In breast cancer cells, STAT5 targets genes that promote cell survival and proliferation including Heat Shock Protein 90-A (HSP90A), IGF and cyclin D1 33. These molecules are also expressed by trophoblast cells and regulate cell cycle; this point should be further investigated.

5.2. MiRNA Expression in trophoblastic cell lines

MiRNAs are considered as factors of epigenetic processes of major relevance during embryogenesis, in special in gamete development, pre-implantation embryo development, and placentation [135]. Additionally, some of their main functions are the control of genes associated with inflammatory responses [136] and regulation of immune tolerance-associated genes, such as HLA-G [137]. Therefore, in recent years, the impact of miRNAs has attracted attention in studies on reproduction and human pregnancy. Many investigations have provided information about miRNA profiles and helped to decipher their function and regulation. However, miRNA landscape in reproduction is still incipient and further investigations are necessary.

In several publications we have reviewed the knowledge on miRNA, including their potential role in evolution. For instance, microRNAs ascended early in evolution, causing that processes of biogenesis and target recognition differ significantly between organisms like plants and animals. For example, only miR-100 is found to be shared by all major animal groups except sponges [138]. The investigation on miRNA evolution led to the proposal of three bursts of miRNAs. One of them based in the bilaterian lineage, the second in the vertebrate lineage and, finally, a third miRNA expansion in the lineage leading to eutherian species. These events generated a correlation between the morphological complexity of a given species and the number of encoded miRNA genes in its genome. To the best of our knowledge, thus far, according to the public miRNA database miRBase, the genome comprises more of 358 miRNA genes for zebrafish, 720 in mouse and more of 1424 in human [139]. However, new miRNA data is accumulating rapidly, but validation of target genes is far from being completed [67].

To elucidate the mechanisms involved in miRNA evolution and to infer their functionality, it requires the recogniton of homologous sequences within species and the extrapolation of large datasets of miRNAs and targets is imperative. Depending on how miRNA-mRNA interactions affect the organism, three different groups can be distinguished: positive selection, neutral selection and negative selection. The majority of miRNA targets resulted from neutral or negative selection reflected by the fact that their targets are not conserved [140]. By searching for orthologs of human mature miRNAs expressed in the placenta, 10 human-specific-miRNAs and 12 further miRNAs with human-specific seed sequences were

identified [67]. Interestingly, most of these miRNAs exhibit low expression but miR-941 showed remarkable abundance in different tissues. Many studies have been performed on miR-941 and revealed its expression in primary first trimester trophoblast cells and its absence in third trimester cells. Additionally, miR-941 is expressed in trophoblast cell lines of first trimester, but not by ACH-3P cells, which is a hybrid of choriocarcinoma cells with first treimester trophoblast. However, the specific effects of miR-941 in the placenta are still unknown [67].

The expression of numerous placenta-associated miRNAs can be mainly localized within the clusters C14MC, C19MC and miR-371-3. It may be hypothesized that the evolution of eutherian species was facilitated by C14MC and that C19MC which may be related with human primate embryo development [80, 141]. The information published in our reviews, revealed that these clusters are conserved in mammals only [142] and are highly expressed in human embryonic stem cells (ESCs). They are essential for cell cycle maintenance [143] and for regulation of proliferation and apoptosis [144].

As mentioned before, some C14MC miRNAs are relevant for the development of the placenta. Some of them have been conserved in all placental mammals, others are restricted to smaller or larger groups of species. In this study miR-134 belonging to C14MC was also investigated. MiR-134 is specifically associated with pregnancy since it is high expressed in placental tissue and in umbilical cord but it is low expressed in nonpregnant women [85]. Our results show that inhibition of miR-134 significantly suppresses cell invasion whilst miR-134 overexpression decreases proliferation only in HTR-8/SVneo cells which suggest an important function during placenta development. However, its specific functions in controlling trophoblast cells should be further investigated.

These placental miRNAs may have the potential for becoming novel biomarkers for prediction and detection of pathologies in pregnancy. For this reason, it is of crucial importance to fully understand the expression patterns in all pregnancy-disorders including preeclampsia, gestational diabetes, intrauterine growth restriction and low birth weight. Having the respective information it may be possible to generate tools for the prevention or treatment of such diseases.

Although the scenario is promising, the search for potential novel biomarkers is hallmarked by limitations and problems. The incipient information about miRNAs and the differences between *in vivo* and *in vitro* models leads to a delay in the process of drug development. Further, the inappropriate technology for the modulation of miRNA expression and hurdles such as drug delivery to the right organs or tissues are some of the problems we are facing. Despite these hurdles, many investigations have been successful, and therapeutic programs are progressing and indicate the potential of miRNAs for diagnosis and therapy of various human diseases [103].

Expression of miRNAs in placental tissue changes dynamically with the gestational age [73, 74, 101, 145] and this change is reflected in vesicles released by several cell types including trophoblast cells [71, 99]. Among others, miR-141 is considered a pregnancy-associated miRNA because it is found at concentrations 10-fold higher in placental tissue than in maternal plasma, its expression increases constantly through pregnancy, and 24 h post-delivery, it cannot be longer detected in maternal plasma [100]. Recently, miR-141 has been detected in trophoblast extracellular vesicles suggesting an important role in the communication between placenta and the maternal system [146]. These observations suggest miR-141 as a pregnancy biomarker which may be expected to be dysregulated in pregnancy disorders as PE.

Despite the small sample number, this study included a homogeneous group of severe PE and their counterpart of normal pregnancies (Table 1). In our hands, an up-regulation of miR-141 was observed in preeclamptic placenta compared with those from normal pregnancies. These data agree with previous reports showing up-regulation of miR-141 in placenta and maternal plasma of severe preeclampsia patients compared with normal pregnancies [145, 147].

In the cell lines used as trophoblast models, HTR-8/SVneo and JEG-3 cells, miR-141 was similarly expressed but compared to normal or PE placenta tissues, it was significantly lower. This confirms our previous report, where we have demonstrated lower miR-141 expression in four cell lines when compared with isolated 1st and 3rd trimester trophoblast cells [75] .

Few studies have been published about the influence of miR-141 on functions in trophoblastic cell lines [73]. However, some investigations in other types of cell lines

revealed that miR-141 regulates functions and plays an important role in tumorigenesis or acts as oncogene. For example, miR-141 has been studied in ovarian cancer cells [148, 149], colon cancer [150], renal cell carcinoma [151], breast cancer [152], pancreatic cancer [153], nasopharyngeal carcinoma [154] and gastric cancer [155] but its exact function is still not clarified.

Consequently the contribution of miR-141 to trophoblast cell proliferation and invasion was investigated. Expression of miR-141 was effectively enhanced after transfection with miR-141-mimic and successfully inhibited after transfection with miR-141-antagomir, evidencing that this method allows analysis of gain- and loss-of-function. Our study showed that miR-141-antagomir inhibits the viability of JEG-3 cells, while miR-141-mimic did not further induce viability. This may be due to the constitutive abundance of endogenous miR-141 in the JEG-3 cells whose function cannot be exhacerbated by additional miR-141 expression. A prior study has also reported over 50% decreased in cell viability after miR-141 inhibition which is much higher than the observed in this study. This discrepancy may be explained by the differences between the methods. In our current study, we used Oligofectamine reagent, while in the previous study, the transfection was performed using Nanofectin [73]. In HTR-8/SVneo cells, no significant differences in viability were detectable after miR-141 over-esxpression or inhibition. This difference between our models, JEG-3 and HTR-8/SVneo cells, may be explained by their different origin. HTR-8/SVneo cells have been immortalized by a vector transformation that induces uncontrolled amplification and splicing of viral DNA resulting in a heterogeneous genotype while JEG-3 is a choriocarcinoma cell line without virus-treatment [75].

The results suggest that miR-141 is involved in the viability of trophoblastic cells, and thus, in placental development. However, the results obtained by MTS assays do not correspond with those obtained by BrdU assays. This discrepancy may be due to the distinct analyzed factors: MTS assays analyze mitochondrial functions in contrast to BrdU assays, which assesses BrdU incorporation into newly synthesized DNA. Further studies are necessary for understanding the mechanisms in the different trophoblastic cell lines.

We have shown that inhibition of miR-141 significantly suppresses invasion in both cell lines, JEG-3 and HTR-8/SVneo. Overexpression of miR-141 promotes the invasion only in HTR-8/SVneo cells. Our data are in accordance with those of Zhang and colleagues [154] who

showed that in nasopharyngeal carcinoma (NPC) cells treated with miR-141 inhibitor the number of cells migrating through Matrigel decreased significantly. However, our report is in contrast to other studies suggesting that overexpression of miR-141 suppresses cell invasion and inhibition of miR-141 promotes invasion in pancreatic cancer [153] and gastric cancer [155]. These opposing findings substantiate the hypothesis that miR-141 has a heterogeneous role and cell-specific response.

Despite the fact that the PE etiology is still widely unclear, previous studies indicate that this disorder may be caused by the imbalanced immune system at the maternal–fetal interface [156] affecting the correct development of trophoblast cells [157]. Moreover, studies have shown that PE results in a shift in angiogenesis and anti-angiogenic factors toward a maladaptive placental circulation [158].

Since miR-141 is altered in placentas from preeclamptic women and may influence proliferation and invasion in trophoblastic cells, we decided to evaluate the function of extracellular vesicles (EMVs) containing miR-141. Our results demonstrate that trophoblast cells released exosomes and microparticles containing miR-141 under normal conditions. After transfection with precursor miR-141, HTR-8/SVneo cells released microvesicles containing elevated levels of mature miR-141. This demonstrates that the cells had processed the precursor miR-141 to its mature form through the RNA interference pathway and packed it into microvesicles. This result emphasizes the importance of miR-141 in the intercellular communication and supports its possible use as biological biomarker.

The next step was to evaluate if miR-141-containing EMVs released by trophoblastic cells regulate functions in recipient cells of the same type or if the intercellular communication occurs between cells at a distance and of different origins. We did not detect effects of EMVs on invasion of trophoblastic cell lines despite of using different concentrations (50 or 100µl). This observation suggests that intercellular communication does not occur between similar cells. Therefore, we hypothesize that trophoblast-derived EMVs affect other cell types. As numerous studies reporthigh levels of circulating EMVs (especially from T cells) in plasma of preeclamptic women [109] and trophoblast-derived EMVs contain miRNA and display immunosuppressive properties [159], we decided to analyze their effectson human T lymphocytes (Jurkat). Our data show a decrease in cell proliferation of T cells in presence of MPs and EXO confirming the hypothesis that intercellular communication by transferring

microRNA frotm trophoblast cells to immune cells occurs. In addition, we observed a cytotoxic effect induced by high concentrations of trophoblast EMVs.

Chapter 6/ Conclusions

EGF plays an important role in regulation of trophoblast. Our data demonstrate that activation of STAT5 in response to EGF promotes cell viability and invasion in trophoblastic cell lines. Understanding the cellular processes induced by this factor might provide relevant information for better understanding various pregnancy-associated diseases. However, we face the problem of selecting cell lines as models for the study of human embryo implantation and placentation.

Numerous members of the miRNA clusters C14MC and C19MC or the miR-200 family can be detected in maternal serum and seem to reflect the state of the placenta potentially having the capacity of becoming novel biomarkers indicating pregnancy disorders. Targeting or mimicking these miRNAs may lead to novel therapeutic concepts.

Alteration in cellular functions after the inhibition or overexpression suggests that miR-134 may play an important role in the control of cell proliferation and invasion of trophoblast cells during the first trimester of pregnancy.

MiR-141 has regulatory functions in trophoblastic cells and is upregulated in PE It is packed into to extracellular vesicles (EMVs) which serve for intercellular communication with distant cells, including immune cells. This communication may contribute to the materno-fetal immunotolerance. Overall, theresults indicate an essential role of miR-141 and EMVs in pathological and physiological conditions in pregnancy.

Chapter 7/ Summary

Trophoblasts are key cells in the development of mammal placenta. One of their functions in humans is anchoring the fetal compartment in the maternal decidua which is achieved by the invading trophoblast cells. This invasion is limited in space and time and its disorders may lead to several pregnancy complications. Previous investigations have revealed that trophoblast invasion is strongly influenced by cytokines and growth factors. One of the most relevant is Epidermal Growth Factor (EGF), which is able to influence positively or negatively a variety of fundamental cell properties. Growth factors and cytokines trigger their effects through several transcription pathways such as Extracellular-signal Regulated Kinase (ERK) and the Janus Kinase-Signal Transducers and Activators of Transcription (JAK/STAT). Although several studies have demonstrated the role of EGF and STAT5 in the regulation of trophoblast behavior, the interaction between both molecules has not yet been investigated. Recent studies have demonstrated STATs are also regulated by microRNAs (miRNAs). Increasing evidence demonstrates that miRNAs are packed and released by trophoblast cells in extracellular membrane vesicles (EMVs) which appear to mediate intercellular communication. Some miRNAs are expressed abundantly in normal placenta and their dysregulation may be involved in the development of pregnancy diseases like preeclampsia (PE).

Aim of this study was to examine the activation of STAT3 and STAT5 by EGF in different trophoblastic cell lines and the effects on cell proliferation, viability and invasion. Additionally, the functions of miR-134 and miR-141 in controlling proliferation and invasion of trophoblastic cell lines should be investigated. The expression of miR-141 should be analyzed in trophoblastic EMVs as well as their effects on immune cells. Finally, the concentration of miR-141 in tissues of normal and preeclamptic placentas was analyzed.

The functions of different cell types have been investigated mainly by MTS- (viability), BrdU- (proliferation) and Matrigel (invasion) assays. RNA and miRNA expression has been analyzed by RT-qPCR, protein expression by Western blotting. STAT5 and miRNAs have been chemically inhibited. MiRNAs have been overexpressed by transfection with their precursor form. EMVs have been isolated from JEG-3 and HTR8/SVneo immortalized first trimester

trophoblast cells and analyzed by NanoSight and RT-qPCR. For analyses of primary cells and tissues placentas from normal and PE pregnancies have been used.

Isolated trophoblasts express high levels of STAT5B, which is similar to HTR8/SVneo cells. Expression in JAR choriocarcinoma cells was significantly lower. EGF-mediated STAT5 activation increased proliferation and viability in both cell lines. STAT5 knock-down resulted in significant decrease of cell viability induced by EGF. Only in HTR8/SVneo cells, invasion decreased after STAT5 silencing and this effect could not be rescued by further addition of EGF. These results demonstrate that STAT5 activated by EGF constitutes an important cascade for the regulation of cell proliferation and invasion in trophoblast cells.

Inhibition of miR-134 significantly suppressed invasion of trophoblast and choriocarcinoma cell lines whilst miR-134 overexpression decreased proliferation only in HTR-8/SVneo cells which suggests a role of miR-134 in placenta development.

MiR-141 was up-regulated in placentas from preeclampsia patients compared to normal pregnancies. Cell lines express similar miR-141 levels and this expression was successfully inhibited or over-expressed by transient transfection. Inhibition of miR-141 resulted in decreased JEG-3 viability and reduced invasion capability of JEG-3 and HTR8/SVneo cell lines. After overexpression of miR-141, HTR-8/SVneo cells secreted EVs with increased miR-141 content. Incubation of isolated EVs with of non-transfected HTR8/SVneo cells did not alter invasion, incubation with Jurkat T cells reduced their proliferation.

In conclusion, the EGF-STAT5 signalling cascade controls fundamental trophoblast functions important for normal placentation. MiR-141 regulates trophoblast invasion and may play an important role in the intracellular communication by a paracrine mechanism based on secretion of extracellular vesicles. It appears also elevated in PE patients and may have potential for serving as a new biomarker.

Chapter 8/ Zusammenfassung

Trophoblastzellen haben Schlüsselfunktionen in der Placenta. Eine dieser Funktionen in der menschlichen Placenta ist die Verankerung des fetalen Gewebes in der mütterlichen Decidua, was durch die Invasion der Trophoblastzellen gewährleistet wird. Die Invasion ist zeitlich und räumlich begrenzt, ihre Störungen können zu verschiedenen Schwangerschaftskomplikationen führen. Frühere Studien haben gezeigt, dass Trophoblastinvasion durch Zytokine und Wachstumsfaktoren kontrolliert wird, wie zum Beispiel durch Epidermal Growth Factor (EGF), der eine Reihe von Zellfunktionen beeinflusst. Wachstumsfaktoren und Zytokine induzieren ihre Effekte über verschiedene intrazelluläre Signalkaskaden wie den Extracellular-signal Regulated Kinase (ERK)- und den Janus Kinase-Signal Transducers and Activators of Transcription (JAK/STAT)-Signalweg. Obwohl eine Beteiligung von EGF und STAT5 an der Regulation von Trophoblastzellen beschrieben wurde, wurden die Interaktionen zwischen beiden noch nicht untersucht. Neuere Studien berichten über die Regulation von STAT-Molekülen durch microRNAs (miRNAs). Es gibt zunehmend Hinweise, dass miRNAs von Trophoblastzellen in extrazelluläre Mikrovesikel (EMVs) eingeschleust werden, die zur interzellulären Kommunikation in die Zirkulation freigesetzt werden. Einige miRNAs sind in der Placenta hoch exprimiert und zahlreiche Schwangerschaftsstörungen, wie die Präeklampsie (PE), gehen mit einer Fehlregulation der miRNAs einher. Bislang wurde jedoch wenig über die konkrete Rolle von miRNAs bei der Regulation fundamentaler Trophoblastfunktionen wie Proliferation, Migration oder Invasion berichtet. Das Ziel dieser Arbeit war die Aktivierung von STAT3 und STAT5 durch EGF in verschiedenen trophoblastären Zelllinien zu untersuchen und die Effekte in Bezug auf Proliferation. Außerdem sollten Funktionen von miR-134 und miR-141 bezüglich der Proliferation und Invasion von trophoblastären Zelllinien untersucht werden. Die Expression von miR-141 sollte in trophoblastären EMVs untersucht werden sowie ihre Effekte auf Immunzellen. Schließlich sollte die Expression von miR-141 in gesunden und PE Placenten verglichen werden.

Die Funktionen der verschiedenen Zelltypen wurden vorwiegend mittels MTS- (Viabilität), BrdU- (Proliferation) und Matrigel- (Invasion) Assays untersucht. RNA und miRNA Expression wurde mittels RT-qPCR gemessen, die Protein Expression mittels Western blotting. STAT5

und die miRNAs wurden chemisch inhibiert. MiRNAs wurden durch Transfektion mit ihrer Precursor-Form überexprimiert. EMVs wurden aus JEG-3 (Chorionkarzinom)-Zellen und HTR8/SVneo (immortalisierte Ersttrimester Trophoblast)-Zellen isoliert und mittels NanoSight und RT-qPCR analysiert. Zur Untersuchung von Primärzellen wurden Plazenten aus gesunden und PE Schwangerschaften genutzt.

Die Expression von STAT5B ist hoch in isolierten Trophoblastzellen, ähnlich wie in HTR8/SVneo-Zellen. Die Expression in JAR-Chorionkarzinom-Zellen war deutlich niedriger. Die EGF-induzierte STAT5-Aktivierung steigert die Proliferation und Viabilität beider Zelllinien. Eine STAT5-Inhibierung bewirkt eine signifikante Reduktion der EGF-induzierten erhöhten Viabilität. Nur in HTR8/SVneo-Zellen ist die Invasivität nach STAT5-Inhibierung vermindert und kann durch Stimulierung mit EGF nicht wieder gesteigert werden. Diese Ergebnisse zeigen, dass die STAT5-Aktivierung durch EGF ein wichtiges Signal für die Regulation der Proliferation und Invasion trophoblastärer Zellen darstellt.

Die Inhibierung von miR-134 supprimiert die Invasivität von HTR8/SVneo-Zellen, während die Überexpression deren Proliferation hemmt. Diese Ergebnisse unterstreichen eine Beteiligung von miR-134 an der Regulation der Placentation.

MiR-141 ist in PE-Plazenten im Vergleich zu gesunden Plazenten erhöht. Trophoblastäre Zelllinien (JEG-3, HTR8/SVneo) exprimieren ähnliche miR-141-Konzentrationen, die chemisch inhibiert oder durch Transfektion erhöht werden konnten. Die Inhibierung von miR-141 bewirkt eine Reduktion der Viabilität von JEG-3-Zellen sowie eine verminderte Invasivität beider Zelllinien. Nach Transfektion von HTR8/SVneo-Zellen mit Precursor-miR-141, haben diese EMVs mit signifikant erhöhten miR-141-Konzentrationen sezerniert, die isoliert werden konnten. Die Inkubation von nicht-transfizierten Zellen mit diesen EMVs beeinflusst nicht deren Invasivität. EMVs mit erhöhter miR-141-Konzentration vermindern die Proliferation von Jurkat T-Zellen.

Es lässt sich schlussfolgern, dass die EGF-STAT5 Signalkaskade für die Plazentation fundamentale Trophoblastfunktionen kontrolliert. MiR-141 reguliert die Trophoblastinvasion und scheint in EMVs verpackt der Kommunikation mit Immunzellen durch parakrine Mechanismen zu dienen. MiR-141 ist in PE Patientinnen erhöht und hat Potenzial diesbezüglich als neuer Biomarker eingesetzt zu werden.

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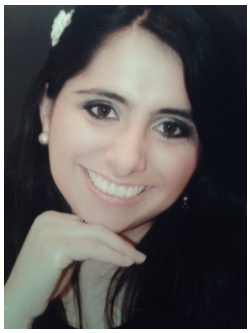
Curriculum Vitae

PERSONAL INFORMATION



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SCIENTIFIC EDUCATION

Post-Graduate Studies	11/2009- 08/2015. PhD Student. Faculty of Biology. Friedrich - Schiller University. Jena, Germany. Thema: Regulation of trophoblast cell functions by intracellular signalling molecules and microRNA.
University Studies	01/2002-03/2008. Department of Biology. Pontificia Universidad Javeriana. Bogotá, Colombia. Thesis: Characterization of the sensibility profile of the cellular line Hep-G2 as a model to establish

cytotoxic activity of some xenobiotic products which need biotransformation by the route CYP450. Final mark: 4.6 highest possible 5.0

Bachelor 01/1990-12/2001. Colegio Minuto de Dios. Bogotá, Colombia.

AWARDS

September 2012 The YW Loke New Investigator Travel Award. JPA/18th IFPA Meeting. Hiroshima, Japan.

August 2010 Travel Award. International Society for Immunology of Reproduction. XI International Congress of Reproductive Immunology. Cairns, Australia.

May 2010 NIH travel Awards. 30th Annual Meeting of the American Society of Reproductive Immunology (ASRI). Pittsburgh, USA.

EMPLOYMENT

2011 - 2013 Laboratory Assistant . Placenta-Labor. Department of Obstetrics, University Hospital Jena, Germany.

SCHOLARSHIPS

March 2014 "Pro-Chance" grant 2014. Friedrich - Schiller University. Jena, Germany. Travel allowance for attending European Congress of Reproductive Immunology. Budapest, Hungary.

February 2013 "Pro-Chance" grant 2013. Friedrich - Schiller University. Jena, Germany. Travel allowance for attending the V Latin American Symposium on Maternal Fetal Interaction & Placenta IV Latin American Chapter of the American Society for Reproductive Immunology. Iguazu Falls, Paraná,

Brazil.

September 2012 "Pro-Chance" grant 2012. Friedrich - Schiller University. Jena, Germany. Travel allowance for attending IFPA meeting 2012. Hiroshima, Japan.

September 2011 "Pro-Chance" grant 2011. Friedrich - Schiller University. Jena, Germany. Travel allowance for attending 2011 Joint Annual Meeting of the German and the Italian Societies for Immunology. Riccione, Italy.

May 2011 "Pro-Chance" grant 2011. Friedrich - Schiller University. Jena, Germany. Travel allowance for attending the Joint International Congress of the American Society for Reproductive Immunology (ASRI) and the European Society for Reproductive Immunology (ESRI). Hamburg, Germany

November 2010 Merck Serono. Arbeitskreis Molekularbiologie der Deutschen Gesellschaft für Gynäkologische Endokrinologie und Fortpflanzungsmedizin. Düsseldorf, Germany.

May 2010 "Pro-Chance" grant 2010. Friedrich - Schiller University. Jena, Germany. Travel allowance for attending the 30th Annual Meeting of the American Society of Reproductive Immunology. Pittsburgh, USA

04 – 09/2010 DAAD PhD Scholarship. German Academic Exchange Program. Jena, Germany.

INTERNSHIP

October 2012 First Reproductive Biology and Immunology Autumn School. Magdeburg, Germany. (1 week).

June 2012 3nd Jena InTReST-DGRM International Training in Reproductive Sciences and Technologies. Placenta-Lab. University Hospital Jena. Jena, Germany. (1 week).

November 2010 2nd Jena InTReST-DGRM International Training in Reproductive Sciences and Technologies. Placenta-Lab. University Hospital Jena. Jena, Germany. (1 week).

LANGUAGES

Spanish Maternal language

English Intermediate

German Basic

CONGRESSES AND MEETINGS

29 March – 01 April 2014	European Congress of Reproductive Immunology Budapest, Hungary Poster presentation
23-28 March 2014	4th Jena InTReST-DGRM International Training in Reproductive Sciences and Technologies. Jena, Germany Organization Committee
18-20 February 2013	V Latin American Symposium on Maternal Fetal Interaction & Placenta IV Latin American Chapter of the American Society for Reproductive Immunology. Iguazu Falls, Paraná, Brazil. Poster presentation
18-21 September 2012	IFPA meeting 2012. Hiroshima, Japan. Poster presentation
3-9 June 2012	3rd Jena InTReST-DGRM International Training in Reproductive

	Sciences and Technologies. Jena, Germany Organization Committee
30 May - 2 June 2012	Joint International Congress of the American Society for Reproductive Immunology (ASRI) and the European Society for Reproductive Immunology (ESRI). Hamburg, Germany. Poster presentation
05 -08 October 2011	First Reproductive Biology and Immunology Autumn School Magdeburg, Germany Oral presentation
28 September -01 October 2011	2011 Joint Annual Meeting of the German and the Italian Societies for Immunology Riccione, Italy Poster presentation
24 -26 August 2011	Joint meeting between the European Society for Reproductive Immunology (ESRI) and the Early Pregnancy Special Interest Group of European Society of Human Reproduction and Embryology (ESHRE) Copenhagen, Denmark Poster presentation
14 -20 November 2010	2nd Jena InTReST-DGRM International Training in Reproductive Sciences and Technologies. Jena, Germany

	Organization Committee
11 -14 November 2010	8th European Congress of Reproductive Immunology.
	Munich, Germany
	Poster presentation
05 -06 November 2010	10. Arbeitskreis Molekularbiologie der Deutschen Gesellschaft für Gynäkologische Endokrinologie und Fortpflanzungsmedizin.
	Düsseldorf, Germany
	Oral presentation
15 -19 August 2010	XI International Congress of Reproductive Immunology.
	Cairns, Australia
	Poster presentation
26 – 28 July 2010	Nanosensor/ Microscopy Workshop
	Jena/Dornburg, Germany
	Assistant
17 - 20 May 2010	30th Annual Meeting of the American Society of Reproductive Immunology.
	Pittsburgh, USA
	Poster presentation
27- 29 May 2008	Morales, D., “Ras in trophoblastic cells and the possible regulative role of microRNAs”. [conference] Reproduction Group, University of Antioquia
	Medellin, Colombia
	Assistant

25-28 September 2007	IX Congreso La Investigación en la Pontificia Universidad Javeriana. Bogotá, Colombia Assistant
10 August 2007	Llerena, A., “Farmacogenética, perspectivas y su aplicación clínica a psicofarmacología” [conference] Universidad Nacional de Colombia Bogotá, Colombia Assistant
26 November – 1 December 2006	II Congreso Colombiano de Zoología Santa Marta, Colombia Assistant
21-24 September 2005	VIII Congreso La Investigación en la Pontificia Universidad Javeriana. Bogotá, Colombia Assistant

List of Publications

Scientific papers

- **Ospina-Prieto S,** Chaiwangyen W, Pastuschek J, Schleußner E, Markert UR, Morales-Prieto DM. STAT5 is activated by epidermal gowth factor (EGF) and induces viability, proliferaton and invasion in trophoblastic cells. Reproductive Sciences. 2015 Apr 9. pii: 1933719115578923.

- **Ospina-Prieto S,** Chaiwangyen W, Markert UR, Morales-Prieto DM. MiR-141 is altered in preeclampsia tissues and regulates trophoblast invasion and intercellular communication. Translational Research (submitted).

- Chaiwangyen W, **Ospina-Prieto S**, Photini SM, Schleussner E, Markert UR, MoralesPrieto DM. MiR-21 regulates trophoblast cell functions by targeting phosphatase and tensin homologue (PTEN) and programmed cell death 4 (PDCD4). Int.J.Biochem.Cell.Biol. (in press).

- Chaiwangyen W, **Ospina-Prieto S**, Morales-Prieto DM, Pereira de Sousa FL, Pastuschek J, Fitzgerald JS, Schleussner E, Markert UR. Oncostatin M and leukaemia inhibitory factor trigger signal transducer and activator of transcription 3 and extracellular signal-regulated kinase 1/2 pathways but result in heterogeneous cellular responses in trophoblast cells. Reprod Fertil Dev 2014 Sep. DOI: 10.1071/RD14121

- Morales-Prieto DM, **Ospina-Prieto S**, Schmidt A, Chaiwangyen W, Markert UR. Elsevier Trophoblast Research Award Lecture: Origin, evolution and future of placenta miRNAs. Placenta. 2014 Feb; 35(28):s39-s45

- Morales-Prieto DM, **Ospina-Prieto S**, Chaiwangyen W, Weber M, Hoelters S, Schleussner E, Fitzgerald JS, Markert UR. Intranuclear crosstalk between Extracellular Regulated Kinase1/2 and Signal Transducer and Activator of Transcription3 regulates JEG-3 choriocarcinoma cell invasion and proliferation. The Scientific World Journal 10/2013; 2013. DOI:10.1155/2013/259845

- Morales-Prieto DM, **Ospina-Prieto S**, Chaiwangyen W, Schoenleben M, Markert UR. Pregnancy specific miRNA-Clusters. J Reprod Immunol. 2013 Mar; 97(1):51.

- Morales-Prieto DM, Chaiwangyen W, **Ospina-Prieto S**, Schneider U, Herrmann J, Gruhn B, Markert UR. MicroRNA expression profiles of trophoblastic cells. Placenta. 2012 Sep;33(9):725-34

- Fitzgerald JS, Abad C, Alvarez AM, Bhai Mehta R, Chaiwangyen W, Dubinsky V, Gueuvoghlanian B, Gutierrez G, Hofmann S, Hölters S, Joukadar J, Junovich G, Kuhn C, Morales-Prieto DM, Nevers T, **Ospina-Prieto S**, Pastuschek J, Pereira de Sousa FL, San Martin S, Suman P, Weber M, Markert UR. Cytokines regulating trophoblast invasion. Advances in Neuroimmune biology (NIB).2012 Jan;2(1):61-97.

Thesis

Ospina S. Characterization of the sensibility profile of the cellular line Hep-G2 as a model to establish cytotoxic activity of some xenobiotics products which need biotransformation by the route CYP450. 2008. Pontificia Universidad Javeriana & Universidad Nacional de Colombia.

Published abstracts

- **Ospina-Prieto S**, Morales-Prieto D, Chaiwangyen W, Schleussner E, Markert UR. Differential expression and function of miR-134 in trophoblastic cells. J Reprod Immuno 101-102 (2014) 53-54.

- Morales-Prieto D, **Ospina-Prieto S**, Chaiwangyen W, Markert UR. Altered miRNA expression in JEG-3 and HTR-8 cells after LIF. J Reprod Immuno 101-102 (2014) 53.

- Chaiwangyen W, **Ospina-Prieto S**, Morales-Prieto D, , Schleussner E, Markert UR. MiR-519d regulates trophoblast cell functions and targets PTEN. J Reprod Immuno 101-102 (2014) 47.

- Markert UR, **Ospina-Prieto S**, Chaiwangyen W, Schmidt A, Morales-Prieto DM. Only humans have human placentas. J Reprod Immuno 101-102 (2014) 10.

- **Ospina-Prieto S**, Morales-Prieto DM, Chaiwangyen W, Schleussner E, Markert UR. Expression and function analysis of miR-141 in trophoblastic cell lines. Placenta, 34 (9), p.A21, Sep 2013.

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